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The gene encoding the neu/erB-2/HER-2 receptor tyrosine kinase is one of a small number of genes known to be altered in human breast and ovarian carcinoma. It is amplified and /or over expressed in approximately one-fourth of these cancers. With its probable role in etiology, and expression at the cell surface, neu has great potential as a prognostic indicator and therapeutic target. Efforts to exploit this receptor in the clinical arena have been impeded by the failure to consider regulation of neu by hormones and co-receptors. this has been complicated by the fact that at least seven different peptide hormones, epidermal growth factor (EGF), transforming growth factor-;alpha, amphiregulin, betacellulin, heparin-binding EGF-like growth, epiregulin, and the diverse neuregulins are all able to activate neu. They work by binding to the co-receptors EGF receptor, erB-3 and erB-4, which then dimerize with neu. Work by this laboratory has shown that each one of these factors activ ates a different constellation of receptors, leading to a great diversity of possible responses. Moreover, the coupling of cellular responses to each factor is determined by the subset of receptors expressed in each tissue. Thus in order to interpret the function of any one of the factors or receptors, including neu, it is essential to consider the influences provided by the entire set.

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FOREWORD

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(5) INTRODUCTION

Amplification and overexpression of the receptor Tyrosine kinase encoded by *HER2/NEU/ERBB-2* is likely to play a significant role in carcinomas where it occurs. However, the presence of activating mutations and agonistic peptide hormones will regulate function of this receptor much more strongly than will receptor abundance. The Type 1 RTKs comprise a network in which the signaling potential of each receptor is conditioned not only by the presence of hormones, but is further regulated by the co-expression of related RTKs. The long-term focus of this grant was to define the capabilities of the ErbB family receptor network: the spectrum of hormones that activate each receptor and receptor combination, and the differences among signalling pathways governed by these receptor systems.

(6) BODY

Breast cancer is one of the most important cancers afflicting women and presents challenging treatment decisions. After p53 alterations, the most frequent change in an identified gene is amplification and/or overexpression of the *neu/ErbB-2/HER-2* gene, which occurs in up to one-third of breast cancers. The gene product (denoted p185 or non-italicized neu), is a receptor tyrosine kinase (RTK) [1-3]. This gene was originally discovered in mutant form in chemically-induced rat nervous system tumors and is now known to be a member of the Type 1, or epidermal growth factor (EGF) receptor gene (*ErbB*) family. The family includes four receptors, which will be referred to herein as the EGFR, neu, ErbB-3, and ErbB-4. (See our review, [4]). Small-scale screens for *neu* alterations in human tumors led to discovery of changes in a number of adenocarcinomas including breast [5], ovarian, gastric, bladder, lung, and colon. Two influential studies showed that *neu*, and not a panel of other oncogenes, is amplified in breast and ovarian carcinomas and that this amplification correlates with RNA and p185 overexpression [6, 7]. Numerous studies of *neu* in mammary carcinoma have led to the following conclusions [4]:

1.*neu* is amplified in 20-30% of mammary carcinomas, with the frequency of amplification higher in tumors from patients with affected lymph nodes [8-16]. The gene amplification suggests

that there is a selection in the tumors for *neu* overexpression. (It cannot be absolutely ruled out that neighboring genes are selected for amplification.)

2.Amplification correlates well with concomitant RNA and p185 expression [6, 7]. An additional 5% of specimens overexpress the receptor without obvious changes in gene structure or copy number [5, 11, 15, 17].

3.There is no evidence for structural mutations in p185 in human tumors. This negative result is weak owing to the high copy number of genes and the large size of the mRNA. Recent work in the mouse transgenic system suggests that this issue should be reconsidered [18].

4.Amplification and/or p185 overexpression can be found in all grades and stages of carcinomas, but not hyperplasia or dysplasia. It is found more frequently in ductal carcinoma in situ (DCIS) than in infiltrating ductal carcinoma (IDC)[14, 16, 19, 20].

5.Amplification and/or p185 overexpression is associated with poor prognosis, especially in node-positive patients. However the extent of this association and independence from other prognostic markers varies greatly among different studies (reviewed, [4]).

Taken together, these data indicate that *neu* amplification and overexpression play a major role in mammary carcinogenesis. This is consistent with findings in model systems: i) the mutated rat *neu* oncogene is as potent as any including *ras* in tissue culture systems. ii)In contrast to other growth factor receptors including the EGFR, overexpression of p185 in the absence of ligand is sufficient to transform cells [21, 22]. iii)Transgenic mice harboring a mutationally activated *neu* oncogene develop multi-focal mammary carcinoma when expressed under control of a murine mammary tumor virus (MMTV) promoter, which confers high level expression in mammary gland and a few other tissues (not found in all studies [23, 24]. iv)Perhaps most compelling is the fact that transgenic mice carrying a structurally normal *neu* gene driven by the MMTV promoter develop metastatic mammary carcinomas [25]. This is noteworthy because it reconstructs what appears to be occurring in human cancer: overexpression of normal p185 in mammary tissue.

Since $p185^{neu}$ is a cell surface protein that seems to play a causal role in mammary carcinogenesis, it is under intensive investigation as a therapeutic target [4, 26]. This work has led to

the validation of HER2/NEU/ERBB2 as a therapeutic target. The Federal Drug Administration has approved the antibody therapeutic "Herceptin" (Genetech) for use on patients with advanced breast cancer. This approval was based upon the excellent performance of this drug and low toxicity in Phase III clinical trials [61, 62]. In spite of the findings linking *neu* to mammary carcinoma, and despite the fact that patients are already being exposed to neu antagonists, at the inception of this grant, little was known about the function of neu either in the organism, or in breast cancer.

The physiological function of p185^{neu,} like any hormone receptor, can only be understood in the context of the hormones that regulate it. The EGFR is activated by binding of at least six different peptide hormones, EGF, TGF-α, amphiregulin (AR), betacellulin (βC), epiregulin [27](epi) and heparin-binding EGF-like growth factor (Hb-EGF)[28-31]. p185, by itself, cannot bind or be activated by these hormones (epi has not been tested). We discovered that EGF and TGF-α, which do not bind to p185, activate p185 Tyr phosphorylation and stimulate p185-associated kinase activity [2, 32]. This phenomenon, now termed **transmodulation**, is dependent upon the co-expression of the EGF receptor with p185 [33-36]. It probably occurs at least in part through formation of receptor heterodimers [37, 38]. Transmodulation of neu by the EGFR is biologically relevant since it works with EGF, TGF-α, betacellulin (see below) and AR, stoichiometrically activates p185 [32], permits association of substrates [39], and correlates with *in vivo* synergy in transforming ability of these two receptors [40]. Thus wherever the two receptors are co-expressed, EGFr agonists activate *neu* . In cell lines that express both receptors, EGF-regulated neu signaling is at least as important as signaling by the EGFR [41]. Since p185 and the EGFR have distinguishable signaling activities [42], this means that regulation of neu production provides a means to alter the signal coupled to EGF.

The transmodulation of neu by the EGFR is a prototype for other interactions within the Type 1 receptor family discovered more recently. Ignorance of these interactions has confused many groups studying the EGFR and resulted in a rather muddy literature which is just now being rationalized [4, 43]. For example, several laboratories independently identified an activity termed Heregulin, neu diffentiation factor, gp30, p75, neuregulin, ARIA, and Glial Cell Growth Factor

(GGF) [44-51], a family of related proteins evidently produced by alternate splicing [50](They will be referred to collectively here as NRG, for the composite name neuregulin, or as NDF). At first the NRGs seemed to be neu ligands since they activate p185 tyrosine phosphorylation in the absence of the EGFR and could be cross-linked to neu [45, 49]. However, it is now known that NRGs bind to both ErbB-3 and ErbB-4 which can then activate neu by transmodulation [52, 53]. A further complication of this receptor system is that ErbB-3 lacks robust kinase activity, and itself requires a second receptor for activity [54].

Additional candidates for neu ligands have been identified but not yet expressed in recombinant form and tested for activity [55, 56]. Nonetheless, the independent purification of NRGs by three different laboratories seeking the *neu* ligand suggests that in mammary epithelia the significant inputs to *neu* may come through transmodulation: transmodulating agonists TGF-α, AR, and NDFs are often produced in mammary tissue or cell lines [57-59] as are the cooperating receptors. Even if these hormones are uniquely responsible for neu activation in mammary tissue, the biological complexities may be enormous. TGF-α and AR, although both EGFR agonists, have somewhat different biological activities [28]. NRGs at first seemed to have radically different activities than EGF agonists since they promote differentiation in some cell lines [49, 60] (but not others [45, 50]), but this has still not been verified in tissue [4].

In summary, *neu* amplification and overexpression is likely to play a significant role in carcinomas where it occurs. However, the presence of activating mutations and agonistic peptide hormones will regulate *neu* function much more strongly than abundance. The Type 1 RTKs comprise a network in which the signaling potential of each receptor is conditioned not only by the presence of hormones, but is further regulated by the co-expression of related RTKs. The long-term focus of this grant is to define the capabilities of the ErbBfamily receptor network: the spectrum of hormones that activate each receptor and receptor combination, and the differences among signalling pathways governed by these receptor systems.

In our view, Herceptin represents the first in a series of pharmaceuticals that will target this receptor, and will become increasingly important in treatment of breast cancer. Further devleopment

and and application of these pharmaceuticals will be aided by the fundamental knowledge about HER2/NEU regulation funded by this grant.

These objectives were:

Aim 1: Signalling of individual receptors and receptor combinations will be compared by investigating receptor phosphoryaltions and substrate phosphorylations to determine how receptor interactions modulate signalling specificity.

Aim 2: Functions of NRG ecto- and endo-domains will be analyzed.

Aim 3: Biological activity of NRGs and NRG/TGF-α combinations will be determined in tissue culture.

Aim 4. Biological Activity of NDFs and NDF/TGF-α combinations in animal models.

Ligands	Receptor Binding and Activation			
	EGFR ErbB HER	ErbB2 HER2 Neu	ErbB3 HER3	ErbB4 HER4
EGF (epidermal growth factor) urogastrone [63]	++			
TGF-α (transforming growth factor α)[30]	++			
AR (amphiregulin)[64]	++			
NRG-1 (neuregulin 1) α and β forms ARIA (acetyl choline receptor inducing activity) GGF (glial growth factor) gp30 HRG (heregulin) NDF (Neu differentiation factor) SMDF (sensory and motor neuron derived factor)			++	++
NRG-2 (neuregulin 2) Don-1 [65, 66]			++	++
NRG-3 (neuregulin 3) [67]				++
BTC (betacellulin) [29]	++			++
EPR (epiregulin)[27]	++			+
HB-EGF (heparin-binding EGF-like growth factor) [68]	++			+

Table 1. Summary of receptor binding and activation, mostly from this study.

Cell Line	NRG		EGF		β-cellulin		AR, HbEGF, and TGF-α[c]
	Tyr Phos of IP'd Receptor[a]	IL-3-independent growth[b]	Tyr Phos of IP'd Receptor	IL-3-independent growth	Tyr Phos of IP'd Receptor	IL-3-independent growth	
LXSN only	NA	N	NA	N	NA	N	NA
EGFR	-	N	+	S	+	S	+
neu	(+)	S	-	N	-	N	-
ErbB-3	-	N	-	NT	-	N	-
ErbB-4	+	N	-	NT	+	N	-
EGFR + neu	* *	S	+ +	P	+ +	P	+ +
EGFR + ErbB-3	+ +	N	+ +	S	+ +	S	+ +
EGFR + ErbB-4	+ +	P	+ +	P	+ +	P	+ +
neu + ErbB-3	+ +	S	- -	NT	- -	N	- -
neu + ErbB-4	+ +	S	- -	N	+ +	S	- -
ErbB-3 + ErbB-4	+ +	N	- -	NT	- +	N	- -

Table 2. Response of Ba/F3 cell lines to growth factors. (Details [69, 70], **Appendix** and unpublished). [a]- and + refer to stimulation above basal PTyr. *ambiguous with high basal PTyr. NA, not applicable; NT, not tested. [b]N-no survival; S, survival, P, proliferation in the presence of the factor designated. [c]The Tyr phosphorylation pattern induced by these three factors was identical; growth responses were not determined.

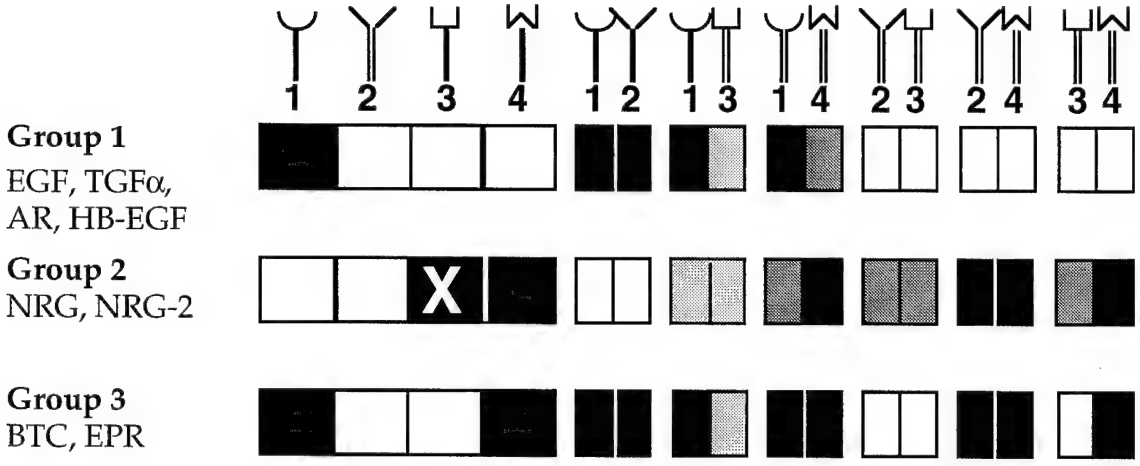

Figure 1. Three distinct functional groups of EGF family hormones.
The patterns of ErbB family receptor activation stimulated by various EGF family hormones are shown for cell lines expressing ErbB family receptors singly or in pairwise combinations. Where there are quantitative variations within groups, the pattern shown applies to the prototype member listed first. A filled box indicates receptor activation, with the fill darkness indicating the intensity of receptor activation. A white "x" on a filled background indicates that the hormone binds the receptor but does not activate it.

The foremost objective for year 1 was to determine the ability of each receptor and receptor combination to respond to each of the EGF family agonists. *Without knowing which hormones activate which receptors, it is impossible to interpret the biological function of either.* We undertook a parallel analysis of the aggregate signaling potential of this receptor family by expressing all four human ErbB family receptors, singly and in each pairwise combination in a uniform cell background. We used the resulting cell lines for the first comprehensive evaluation of ErbB family receptor activation and coupling to cellular responses by NRG, NRG-2, EGF, Betacellulin, AR, TGF-α, HB-EGF, and epiregulin. (Table 1, 2, and Fig. 1). This work has resulted in seven publications [66, 69-74] and completes Tasks 1a, 1b, and 1c. In part, because the scope of the work grew as more ligands were discovered, we were unable to complete Task 1d.

A. Approach. The mouse Ba/F3 pro-B-lymphocyte cell line was chosen for expression of ErbB family members because the survival and proliferation of the cells are tightly regulated by exogenous growth factor (IL-3). Prior to the onset of this grant we began to produce cell lines by transfection of all four ErbB family cDNAs singly and in each of the pairwise combinations. The response of cells to these factors was measured in two ways: i.) the Tyr phosphorylation of the receptor(s) expressed in the cell lines was determined by immunoprecipation and anti-PTyr

immunoblotting. These results define the ability of each factor to couple to each receptor, and in essence define for the first time the full capabilities of the ErbB family factor and receptor network. ii.)The ability of the ligand-activated receptors to enable IL-3-independent survival and proliferation of these lines was determined. These data reveal the degree to which combinatorial interactions govern the growth responses. A brief summary follows, with greater depth provided in the corresponding references [66, 69-74]. Data are summarized in Tables 1,2, Fig. 1.

B.Single expressing cell lines. (Table 1, Table 2). NRG stimulated Tyr phosphorylation of ErbB-4 but neither the EGFR nor ErbB-3. ErbB-3 binds NRG, but does not become Tyr phosphorylated owing to its weak intrinsic kinase activity [54]. The ability of neu on its own to respond to NRG probably occurred through transmodulation via the limiting amount of endogenous ErbB-3. The β-cellulin results are noteworthy in demonstrating for the first time that a single growth factor can activate both the EGFR and an additional receptor. Later work by our lab extended this pattern of responses to epiregulin [71], and by another lab to HbEGF [75]. Each factor activates a characteristic combination of single receptors: For example, β-cellulin activates the EGFR and ErbB-4 equally well, whereas EGF is specific to the EGFR, and NRG is specific to ErbB-3 and -4. NRG-2 binds to ErbB-3 and ErbB4, and Epiregulin strongly activates the EGF receptor, but weakly activates ErbB4. Of all the EGF-related factors tested, only Cripto-1 doesn't activate any of the receptors. Responses of ErbB4 to Betacellulin and to Epiregulin are greatly enhanced in the presence of ErbB-2.

C. Transmodulation in double expressor cell lines (Fig. 1)**.** This work showed for the first time the unanticipated promiscuity of this network, and specifically, that NRG can regulate Tyr phosphorylation of the EGFR, if ErbB-3 or ErbB-4 are present, and that NRG-responsiveness of ErbB-3 can be conferred by co-expression of any of the EGFR, neu, or ErbB-4. Thus NRG promotes extensive interactions of ErbB-3 and ErbB-4 with the other receptors. Similarly, EGF and β-cellulin promote extensive cross-talk. The interactions do show some specificity, however, and additional specificity may be masked by variable receptor levels. The greatest specificity was seen with the ErbB-3/ErbB-4 combination: NRG stimulated Tyr phosphorylation of both receptors, but

β-cellulin stimulated only ErbB-4. Thus there are qualitative as well as quantitative specificities in transmodulating interactions.

D.Coupling to IL-3 independent growth. Although the specific responses of Ba/F3 cells will not necessarily predict those in other backgrounds, they reveal the extent to which combinatorial receptor interactions govern these responses. With a single exception, activation of either neu or the EGFR by any of the peptides was associated with cell survival. In contrast, ErbB-3 had no apparent influence beyond enabling NRG to activate EGFR and neu. ErbB-4 failed to promote survival or proliferation on its own or in concert with ErbB-3. Il-3-dependent proliferation was only seen with activation of certain receptor combinations: EGFR + neu, and EGFR + ErbB-4.

Task 1b was to survey substrate phosphorylations in activated Ba/F3 cells. This has been done using phospho-tyrosine immunoblots to identify growth factor-regulated phospho-proteins. The spectrum of phosphorylated substrates correlated with the particular receptor activated, rather than the particular growth factor used. Since the survival and proliferation assays suggest that EGFR and ErbB-4 (activation of both was required for proliferation) perform different functions, we have been especially interested in differences between EGFR and ErbB-4 substrates. We have now identified many of the substrates activated by the EGFr and ErbB4, using molecular weights as a guideline, and precipitating antibodies for verification. Of greatest interest has been the small subset that may be ErbB4 specific. One candidate is a 65 kDa substrate that does not seem to be SHP2, Shc, Sam68, Src family members, JNK2, or $p70^{S6K}$. Further work will be needed to identify this peptide, however.

In Ba/F3 cells, EGFR activation was accompanied by the phosphorylation of c-Cbl and Shc, two known signaling effectors for EGFR. In contrast, activated ErbB-4 was not accompanied by c-Cbl or Shc phosphorylation. This suggests that Shc and c-Cbl are downstream signaling effectors for EGFR and not for ErbB-4. In cells expressing both EGFR and ErbB-4, BTC stimulated high levels of EGFR, ErbB-4, Shc, and c-Cbl phosphorylation. However, Shc and c-Cbl preferentially complexed with EGFR and not with ErbB-4, suggesting again that Shc and c-Cbl are signaling effectors for EGFR and not for ErbB-4.

Task 2. Mammary cell lines.

Aim 1, Task 2. Mammary Cell Lines. There is mounting evidence that while activation of either EGFR or ErbB-2 stimulates mammary cell proliferation and promotes tumorigenesis, increased ErbB4 signaling may inhibit proliferation or tumorigenesis by stimulating differentiation. Ectopic treatment of breast tumor cell lines with NRG inhibits their growth and stimulates milk protein synthesis. Moreover, ErbB4 overexpression in human mammary tumor samples correlates with markers for a more favorable prognosis, suggesting that ErbB4 signaling may inhibit tumorigenicity [Bacus, *et al.*, 1996]. In initiating this work, as discussed below, we encountered a major technical problem, the inability to enforce high level expression of ErbB4. This problem, and coverage of some of the other sub-tasks by publications from the Hynes group, prevented completion of this task.

We wished to examine the effects of increased ErbB4 signaling on the proliferation of MCF-10A cells. However, in these cells ErbB4 tyrosine phosphorylation is not stimulated by either NRG or BTC. Therefore, we tried to establish MCF-10A derivatives that ectopically overexpress ErbB4 through infection with a recombinant retrovirus containing the neomycin resistance gene and the human ErbB4 cDNA . We noted that this retrovirus stock had an unusually low titer in MCF-10A cells and that the rare transformants that arose from infections with the ErbB4 retrovirus did not express higher levels of ErbB4 than the parental MCF-10A cells. Retrovirus stocks harboring ErbB-4 showed a much higher ratio of fibroblast/MCF10 colony-forming units than did control retroviruses, and the few infectants recovered failed to express high levels of ErbB-4. This suggests that ErbB-4 is indeed a growth inhibitor of MCF-10A cells, probably through induction of differentiation.

II.NRG Intracellular Domain

Task 3.NDF (JM)

a.aggregation mo. 1-3
b.expression and endo-domain experiments
c.production anti-NDF

Task 3 has been largely completed, although, as discussed below, the experimental story is still incomplete.

The cytoplasmic domain of NRG family EGF-related growth factors is unusual in showing extraordinary diversity of regulation by splicing, and in that some forms have unusually long cytoplasmic tails of unknown function (over 400 amino acids). We hypothesize that these tails are likely to themselves transmit signals so that binding of NRG ecto-domains to the cognate receptors results in bidirectional signaling. As a first step in addressing this problem, we have begun to clone and express these factors in transient and stable transfected cell lines. This work was complicated by some expression problems that were ultimately solved by changing expression vectors. We finally succeeded in expressing full length and cytoplasmic deletion forms of NRG both transiently and stably in NIH 3T3 cells. We are using both myc- and flag- epitope tagged molecules to enable recovery of the proteins by immunoprecipitation and immunoblotting.

Tasks 3a and 3b. Using transient expression of NRG in Cos-7 cells, and stable expression in NIH3T3 cells, we have found that cell surface expression of NRG induces a stable and tight binding of Ba/F3 cells expressing ErbB-4 or ErbB-2 and ErbB-4 to the NRG-expressing cells. Thus membrane anchored NRG may indeed serve as an adhesion receptor. To test our hypothesis that NRG induces a cell-autonomous signal when activated, we analyzed the phenotype of stable cell lines expressing full-length or truncated NRG. It appears that the presence of the cytoplasmic tail is associated with growth inhibition, although this provisional result remains to be quantitatively verified. We did not succeed in using a regulated aggregation system, however, since EGFR/NRG chimeric molecules proved to be unstable.

The major focus of work on the NRG intracellular domain has been to develop physical and two-hybrids screens to identify binding partners. We have now completed the two-hybrids screen using the first cytoplasmic exon of NRG as bait. Approximately 4 X 10^6 transformants were tested, yielding 36 positives that fulfilled our initial and final screening criteria. Two-thirds of these have been sequenced and analyzed by comparison to the nucleci acid and protein data bases. The results were as follows:

Amphiphysin II. Isolated twice.

Enigma (frame-shift).

Myd 88.

4 ribosomal phosphoproteins. These are often encountered as false positives in two hybrids screens.

alpha-Globin. Isolated three times.

β-globin. Isolated three times.

mouse acid phospho-protein.

β-actin

8 different isolates that do not match with known gene sequences.

Triage of positives. We have decided to place greatest emphasis on characterization of Amphyphysin II, which has the appropriate intracellular localization and biological activities to be plausibly connected with the cytoplasmic domain of NRG. This is based on the rationale that the ribosomal phospho-proteins are typical false-positives for two hybrids screens, and that globins and actin are highly expressed, and are likely to be over-represented in the two hybrids library. Enigma was out-of-frame.

Amphiphysin II is a recently identified cytoplasmic protein related to Amphiphysin I. Amphiphysin I binds to dynamin, and has been implicated in vesicle endocytosis and recycling in the nervous system [81]. Both Amphiphysin I and II are broadly distributed. They form heterodimers that seem to be important for clathrin-mediated endocytosis [82]. Hence one hypothesis would be that interaction with Ampiphysin II interactions with NRG are related to ligand or kinase C-regulated endocytosis of NRG. Other information about Amphiphysin II suggests possible links with signal transduction. Amphiphysin II contains an SH3 domain, a constituent of many signaling adaptor proteins. Moreover, amphiphysin II has been found to associate with two proto-oncogene products, Myc and Abl [83,84]. Finally, the assembly of amphyphysin II into complexes associated with the cytoskeleton suggests other possible roles in signaling [84].

In order to characterize these potential interactions further, we constructed epitope-tagged versions of Amphyphysin II and NRG for transient and stable cell lines. We can detect high level expression of both proteins, and have found that they can be co-immunoprecipitated. We are continuing by mapping the sites of interaction. The strategy is to use the two-hybrid assay to map

sites, and confirm with complementary results in the GST-pulldown system. This has enabled us to map the minimal interaction to exon 11, but a larger cytoplasmic domain yields more signal. Finally, mutant activities will be correlated with changes in function associated with these two candidates, as discussed in the original proposal. However, this work has now extended beyond the duration of the original proposal.

Glutathione-S-transferase (GST)-fusion proteins. A complementary method for identification of interacting proteins is affinity purification. This has the advantage of using full-length cellular proteins, that are expressed in their native environment, but probably requires stabler interactions than two-hybrids screening. In order to complete the current work, we are using pull-down experiments with GST-fusion proteins derived from NRG and Amphyphysin II to verify the two-hybrids results. Finally, we will use in vitro experiments, with NRG deletion mutants for loss-of-function and as dominant negatives, in order to determine the function of these interactions. The two major working hypotheses are that NRG interactions with Amphiphysin II are important in intracellular routing of NRG, or, with the ability of Amphiphysin to bind signaling proteins Myc and Abl, that this interaction conveys a NRG signal through one of these protiens.

GST fusion proteins have been produced with GST-coding sequences fused to the three intracellular exons. These proteins have been labeled by in vitro phosphorylation at a protein kinase A acceptor site tag, and used as probes in Far-Westerns. So far, these assays have been noisy, and we are working to clean up the background using an irrelevant WW domain fusion protein as negative control.

We have had success in using tagged GST-fusion proteins for measuring AmphiphysinII/NRG interactions. For example, a GST-NRG exon 11,12,13 (complete cytoplasmic domain) fusion protein can be used to affinity isolate Amphiphysin II from Cos7 cell extracts. Analysis of other fusion proteins is currently underway.

The same fusion proteins have been used to make GST-fusion protein affinity columns for isolation of other NRG- binding proteins. The biggest technical problem has been non-specific protein binding, which we are working to circumvent. In the long run, if the Far-Western's can be

improved, we will use the probe to screen a cDNA library, as discussed in the proposal. Alternatively, clean results in the affinity purification may provide us with material that can be identified using MALDI-Mass Spectrometry.

III.Biological Activities of NRG and TGF-α.

Pellets were impregnated in the mammary fat pad of virgin female mice with NRGα or β, or TGF-α without or with estrogen/progesterone. Implants were inserted into the the surgically-exposed number 4 mammary fat pad of 32 da Balb/c females, with growth factor-free pellets used as controls in the contralateral mammary fat pad. Four days after implantation whole mounts were prepared and stained with hematoxylin.We favor this approach because the epithelium responds in the context of normal matrix and hormonal environment and hence is highly physiological, and has the collateral advantages of being fast, manipulable and inexpensive. Although there is considerable literature for both EGF and TGF-α, the NRGs had not been investigated in this format. These experiments resulted in a publication [76]. There were two exciting results from this work. First, we found that although NRGs and TGF-α can both enhance ductal extension and branching and lobuloalveolar development, only lobuloalveoli induced by NRG α or β contained secretory products. Thus only the NRGs can induce full terminal differentiation. This is consistent with the finding that TGF-α is expressed throughout postnatal mammary development, but NRG is only expressed in pregnancy. A second exciting result was that NRG-α was more potent than NRG-β in these assays. This is a preliminary result owing to the difficulty in ensuring that biological activities of the preparations were matched, but is intriguing: Birchmaier has reported that NRGα, but not NRGβ, is expressed in mammary tissue. On the other hand, in all reports comparing activity of these hormones, they are qualitatively similar, but **NRGβ** is more potent. Hence our new results suggest for the first time a mammary-specific preference of unknown mechanism for responsiveness to NRGα, perhaps even involving an accessory receptor.

(7) KEY RESEARCH ACCOMPLISHMENTS

- Determination of which EGF ligand family hormones regulate which receptors when expressed alone
- Determination of which EGF family hormones regulate which receptor combinations
- Determination of the combinatorial abilities of EGF family hormones to promote survival or proliferation of Ba/F3 cells, revealing synergistic signaling combinations and unique specificities of individual receptors
- Determination that a subset of EGF family ligands yield strong interactions with both EGF receptor and ErbB/4.
- Discrimination of TGF-α versus Neuregulin functions in mammary development
- Identification of candidates for interaction with Neuregulin cytoplasmic domain

(8) REPORTABLE OUTCOMES

manuscripts

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presentations

1995
3/95 U MAss Amherst
3/95 UC Santa Cruz
5/95 NYU Pathology11/95 Mayo clinic

1996
8/96 Peptide Growth Factors Gordon Conference (invited speaker)
11/96 Yale symposium (invited)

1997
2/97 Banbury conference "Heregulins and their receptors" invited speaker
4/97 Mt. Sinai Biochemistry Department invited
4/97 McGill University Dept. of Pharmacology invited
5/97 Endocrine Society Annual Meeting, invited platform
8/97 Gordon Research Conference "Cancer"
9/97 "Modes of EGF Signaling" Iowa State University Growth factor and signal transduction conference invited platform talk
10/97 Era of Hope (USAMRMC) invited platform talk
11/10/97 Wyeth-Ayerst Research invited speaker
Network Interactions of EGF family Receptor Tyrosine Kinases in Breast Cancer
11/19/97 Charlestown/ Cutaneous Biology Research Center
EGF family receptor tyrosine kinases and yeast DNA checkpoint genes in cancer

1998
3/12/98 Pfizer Central Research invited speaker
HER2/Neu/ErbB2 receptor tyrosine kinase in breast cancer
8/15/98
Targeted Expression Of Dominant-Negative *Erbb2/ Her2/Neu* And *Erbb4* In The Mouse Mammary Gland Reveals Functions In Mammary Development
platform talk at
"Tyrosine Phosphorylation and Cell Signaling" The Salk Institute 8/15-19/98
10/28/98 Yale Cancer Center Developmental Therapeutics
HER2/Neu Receptor Tyrosine kinase in breast cancer: new target, new approaches

11/98 Yale Medical School Lung Biology Conferences HER2/Neu/ErbB2 receptor tyrosine kinase in breast cancer

funding applied for

R01CA80065 from NIH was based partly on work funded by this grant, and was awarded funding by the NIH beginning 7/99

employment

David Riese, II, was hired for a faculty position, (Assistant Professor, Dept. of Department of Medicinal Chemistry & Molecular Pharmacology; Purdue School of Pharmacy and Pharmacological Sciences), based largely upon work for this grant

(9) CONCLUSIONS

This grant has produced many novel and significant observations, and has yielded eight publications [66, 69-74, 76]. The definition of the abilities of individual EGF-related hormones to regulate specific receptors and receptor combinations is absolutely essential for understanding the biology of any of these hormones and their receptors and is a significant contribution of this work. This "wiring diagram" will benefit all investigators working in the system and is a unique contribution of our laboratory. The discovery of new EGF-related hormones makes this goal a moving target, but we will continue to fill out this elemental work.

We analyzed the NRG-dependent responses conferred upon the Ba/F3 mouse pro-B-lymphocyte cell line by expression of the four ErbB family receptors, singly and in pairwise combinations. In the presence of appropriate co-receptors, NRG regulated the tyrosine phosphorylation of all four ErbB family receptors. While some of the NRG-induced interactions between ErbB family receptors observed had been predicted from previous work, we demonstrate here for the first time that in the presence of ErbB-3 or ErbB-4, NRG regulates tyrosine phosphorylation of the EGFR, and that the presence of the EGFR, neu, or ErbB-4 enables NRG to regulate tyrosine phosphorylation of ErbB3. Furthermore, NRG induces IL-3-independent survival or proliferation in only a subset of the lines that exhibit NRG-induced receptor tyrosine phosphorylation. Thus the biological responses to NRG are specified at several different levels of regulation.

Previous analyses of NRG-induced signaling by ErbB family receptors have been carried out with a few receptor combinations in a variety of cell backgrounds. In mammary cells, NRGs induce neu tyrosine phosphorylation, can be cross-linked to neu, and binding is increased by neu overexpression, at first suggesting that neu is a receptor for NRG. However, NRG fails to induce neu tyrosine phosphorylation and/or bind neu when neu is expressed in fibroblasts, ovarian cells, CHO cells , T-lymphoid cells, or COS-7 cells. Moreover, NRG binds ErbB-3 or ErbB-4 , and co-expression of ErbB-3 or ErbB-4 with neu confers NRG responsiveness upon neu. This has led to the general working hypothesis that activation of neu by NRG requires the presence of ErbB-3 or ErbB-4. The present data are compatible with this conclusion, and extend the model to include NRG regulation of the EGFR. The EGFR and ErbB-3 alone fail to respond to NRG for two different reasons. The EGFR does not bind NRG, whereas ErbB-3 binds, but is impaired for kinase activity. The stimulation of tyrosine phosphorylation of neu by NRG might suggest direct activation of neu by NRG, but in view of previously published work, is more likely to reflect interaction with endogenous ErbB-3. However, ErbB-4 is able to bind and respond to NRG directly.

NRG induces extensive cross-talk among receptors expressed in binary combinations (Table 2). Either ErbB-3 or ErbB-4, both of which bind NRG, enable regulation of the EGFR by NRG. This is the first evidence that NRG can regulate EGFR signaling. As predicted from earlier work, NRG stimulates tyrosine phosphorylation of both receptors in the neu + ErbB3, neu + ErbB-4, and ErbB-3 + ErbB-4 cell lines. Coexpression of EGFR, neu, or ErbB-4 with ErbB-3 permits NRG induction of ErbB-3 tyrosine phosphorylation. Although earlier work showed that expression of neu enhances tyrosine phosphorylation and NRG regulation of ErbB-3 [Carraway, *et al.*, 1994], that work was done in COS-7 cells, which express significant basal amounts of neu and EGFR. Thus the present work demonstrates for the first time that *de novo* expression of either the EGFR, neu, or of ErbB-4 enables hormone-regulated phosphorylation of ErbB-3. Endogenous ErbB receptor expression in Ba/F3 cells played a limited, yet significant role in specifying responses to NRG stimulation in these experiments. While endogenous ErbB-3 expression permits NRG stimulation of exogenous neu tyrosine phosphorylation, NRG does not stimulate receptor tyrosine

phosphorylation in cells that express exogenous EGFR only. Perhaps the level of endogenous ErbB-3 expression in Ba/F3 cells is insufficient to permit NRG-induced EGFR tyrosine phosphorylation. Alternatively, intrinsic differences between the exogenous human and endogenous mouse proteins may result in the differing capacities to undergo NRG-induced heterotypic receptor interactions.

While NRG can stimulate the tyrosine phosphorylation of each receptor under the appropriate conditions, the diversity of biological responses to NRG indicates that there must be additional mechanisms by which biological responses to NRG are specified. The patterns of NRG-induced stimulation of ErbB receptor tyrosine phosphorylation and IL-3 independent survival or proliferation demonstrates that there are several hierarchical levels at which biological responses to NRG are apparently specified.

First, responsiveness to NRG requires the expression of ErbB-3 or ErbB-4. Previous work and results presented here establish that NRG can not bind or stimulate tyrosine phosphorylation of ErbB family receptors in the absence of ErbB-3 or ErbB-4 expression. Biological responses to NRG are also specified by the intrinsic kinase activity of the ErbB family receptor(s) stimulated by NRG, since the kinase-deficient ErbB-3 requires the presence of a co-receptor for hormone-regulated phosphorylation.

Distinct biological responses to NRG are also conferred by ligand-induced coupling of different ErbB family receptors to different signaling pathways. NRG enables the IL-3 independent survival of cell lines expressing neu, probably through NRG-induced activation of neu via ErbB-3. However, NRG does not enable the IL-3 independent survival of EGFR + ErbB-3 cells, or of ErbB-4 cells, even though NRG stimulates receptor tyrosine phosphorylation in these lines. This demonstrates that neu has signaling properties distinct from those of the EGFR, ErbB-3, or ErbB-4, and is consistent with earlier work showing that different ErbB family receptors can activate different signaling pathways and responses. Neither NRG nor EGF induces IL-3-independent proliferation of cells that individually express ErbB-4 or EGFR (Table 1). Yet, NRG stimulates IL-3-independent proliferation in the EGFR + ErbB-4 cell line. One simple explanation would be that IL-3-

independent proliferation requires activation of two independent pathways, one of which is activated by the EGFR, and one by ErbB-4. An interesting alternative would be that EGFR and ErbB-4 phosphorylation sites differ in ligand-induced EGFR/ErbB-4 heterodimers than in ligand-induced receptor homodimers owing to substrate specificity of the receptor catalytic domains and steric considerations in the cross-phosphorylation reaction. This would permit recruitment of unique signaling proteins to the heterodimer, resulting in unique biological responses. Thus, the diversity of hormone-regulated outputs from this receptor network may extend beyond the simple combinatorial possibilities.

Since EGF and NRG bind to different receptors, it can be predicted that individual members of the EGF family of ligands activate different constellations of ErbB family receptors, so that these different ligands will yield distinct patterns of biological responses. Evidence presented here supports this prediction. Previous reports demonstrated that betacellulin binds to the A431 human adenocarcinoma cell line and the MDA-MB-453 human breast carcinoma cell line, both of which overexpress the EGFR. This binding was quenched by the addition of an excess of EGF, suggesting that betacellulin is a ligand for the EGFR. However, the effect of betacellulin on EGFR tyrosine phosphorylation and signaling and the possibility that betacellulin might activate other ErbB family receptors were not assessed. We have shown that in Ba/F3 cells expressing only a single ectopic ErbB family receptor, betacellulin stimulates the tyrosine phosphorylation of both the EGFR and, surprisingly, ErbB-4 (Table 1). This is consistent with the observation that radiolabeled betacellulin binds specifically to EGFR and ErbB-4, but not to neu (Plowman, *et al.*, in preparation). Control experiments performed in parallel demonstrated that radiolabeled amphiregulin and EGF bound only to EGFR and radiolabeled NRG-β bound only to ErbB-4, as previously reported. Thus, betacellulin exhibits activities that are distinct from those displayed by EGF, which activates the EGFR alone, and NRG-β, which activates ErbB-3 and ErbB-4 (Table 1). Furthermore, in this first comprehensive analysis of ErbB family transmodulation for both betacellulin and EGF, we find that EGF can transmodulate ErbB-4 in the EGFR + ErbB-4 cell line (Table 1). We also demonstrate that

betacellulin stimulates a pattern of receptor transmodulation that is qualitatively distinct from the patterns stimulated by EGF and NRG-β.

With one exception, betacellulin, EGF, and NRG-β transmodulated the tyrosine phosphorylation of all four ErbB family receptors in cell lines that express any receptor for each ligand (Table 1). Thus, differences in ligand activities in the double recombinant cell lines can be predicted by differences in activities in the single recombinant cell lines. For example, NRG-β activates ErbB-3, while betacellulin does not activate neu or ErbB-3. Not surprisingly, in cells expressing neu + ErbB-3, NRG-β stimulates the phosphorylation of both receptors, while betacellulin does not stimulate the phosphorylation of either receptor in this cell line (Table 1). The single exception is the response of the ErbB-3 + ErbB-4 (3+4) cell line to betacellulin. Both betacellulin and NRG-β stimulate ErbB-4 tyrosine phosphorylation in the single recombinant cell line. However, in 3+4 cells, NRG-β stimulates the tyrosine phosphorylation of both receptors, while betacellulin stimulates the tyrosine phosphorylation of ErbB-4 but not of ErbB-3 (Table 1). Nonetheless, because NRG-β binds ErbB-3, it is not clear that this absence of ErbB-3 tyrosine phosphorylation is due to differences between betacellulin- or NRG-β-induced ErbB-3 transmodulation.

Previous work demonstrated that different ErbB family receptors or combinations of receptors couple to distinct cellular signaling pathways. For example, EGFR activation stimulates the tyrosine phosphorylation of four proteins that are not highly phosphorylated following neu activation [77]. Furthermore, activated ErbB-3 stimulated higher levels of phosphatidylinositol 3-kinase than EGFR did [78-80], and it has been suggested that EGFR and neu bind the adapter protein GRB2, but ErbB-3 does not (Prigent and Gullick, 1994). These different coupling capacities of the ErbB family receptors can be correlated to specific biological responses. In Ba/F3 cells, activation of neu stimulates IL-3 independent survival, while activation of EGFR and ErbB-4 together stimulates IL-3 independent proliferation .

We found that betacellulin stimulates IL-3 independent survival or proliferation in neu + ErbB-4 cells and in every cell line that expresses EGFR. In contrast, EGF stimulated IL-3

independence only in those cell lines that express EGFR while NRG-β stimulated IL-3 independence only in the EGFR + ErbB-4 cell line and in those cell lines that express neu (Table 1). Therefore, with a single exception, the minimal requirement for IL-3 independence is activation of either EGFR or neu. The exception is that betacellulin and EGF, but not NRG-β, stimulated IL-3 independent survival in the EGFR + ErbB-3 cell line (Table 1). This lack of response to NRG-β may merely reflect the lower level of EGFR phosphorylation stimulated by NRG-β in this cell line. On the other hand, the absence of biological response may reflect the different mechanism by which the EGFR is activated. We demonstrated that coupling of these multiple receptor species to cellular signaling pathways acts in a non-additive manner in specifying biological responses (Table 1). Accordingly, while activation of EGFR or neu by themselves stimulated IL-3 independent survival, activation of EGFR and either neu or ErbB-4 together stimulated IL-3 independent proliferation (Table 1).

Our data suggest that differences in NRG-β, EGF, and betacellulin activities play a significant role in specifying the proliferation and differentiation of human tissues *in vivo*. Increases in the expression and/or signaling of ErbB family receptors plays a significant role in tumors of mammary or neuroectodermal origin [4]. Because betacellulin, NRG-β, and EGF have distinct biological activities that apparently reflect their differing abilities to activate receptor signaling, it may be possible to develop antagonists that specifically disrupt signaling by a single EGF family ligand and may inhibit the genesis or growth of malignancies without disrupting the activity of other EGF family ligands in the same tissue.

Our work also broke ground in discriminating the biological functions of TGF-α and NRGs in mammary tissue. Thus for the first time we can definitively identify a truly physiological differentiative response with NRGs. Moreover, the possibility that mammary tissue has a privileged response to NRGα over NRGβ raises many interesting possibilities.

We have also made progress on identifying a function for the NRG cytoplasmic domain. It appears to inhibit growth of cells, either by inducing apoptosis, or by inhibiting proliferation. Thanks to our screening efforts, we have identified an important intracellular routing protein that may be

involved in growth regulation, Amphiphysin II, as a candidate interactor with the NRG tail. Ongoing work is devoted to determining the consequences of this interaction.

The difficulties in engineering MCF-10A cells to express ErbB-4 impeded some of the original experiments proposed for task 2, but answer a major biological question that these experiments were to address, namely the differences in cellular responses coupled to each of the receptors in mammary backgrounds. If we find, as we suspect, that the growth inhibition is due to differentiation, the work will be completely consistent with the implant experiments, and will provide strong evidence that the NRG/ErbB-4 couple is a major regulator of mammary differentiation. This has implications for breast cancer patients, since active ErbB-4 would be associated with a favorable prognosis, and may suggest hormone therapies based on NRG, or other ErbB-4 activators.

We are continuing to approach this problem using the mouse mammary cell line HC11. HC11 cells are a Balb/c mammary cell line cultured from a mid-pregnant female that can be induced to differentiate and activate milk protein synthesis (β-casein) in the presence of dexamethasone, insulin and PRL (DIP). In order to acquired DIP responsiveness, HC11 cells must first become "competent" through confluent incubation in the presence of EGF. However, EGF actually suppresses induction of β-casein by DIP. This latter response is mediated by Stat5. In contrast to EGF, mutationally activated ErbB-2 induces competence, but has no inhibitory effect on β-casein. Finally, NRG resembles EGF in inducing competency, and inhibiting β-casein production, and inducing production of a 22kd milk protein in the presence of DIP. These EGF-like properties and failure to induce full differentiation by NRG contrast with results of pellet implant experiments. Although there are numerous differences between the in vitro and in vivo situations, there may be a simple explanation. We have found that HC11 cells express EGFR, ErbB-2, and ErbB-3, but do not produce ErbB-4 mRNA detectable by RNase protection. We hypothesize that introduction of ErbB-4 into these cells will convert the NRG response to a differentiation response, and are currently testing these idea as an alternative approach to Task 2.

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The Cellular Response to Neuregulins Is Governed by Complex Interactions of the erbB Receptor Family

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Deregulated signaling by the four members of the epidermal growth factor receptor tyrosine kinase family (erbB family) is implicated in the genesis or progression of human cancers. However, efforts to analyze signaling by these receptors have been hampered by the diversity of ligands and extensive interreceptor cross talk. We have expressed the four human erbB family receptors, singly and in pairwise combinations, in a pro-B-lymphocyte cell line (Ba/F3) and investigated the range of interactions activated by the epidermal growth factor homology domain of the agonist neuregulin β. The results provide the first comprehensive analysis of the response of this receptor family to a single peptide agonist. This peptide induced complex patterns of receptor tyrosine phosphorylation and regulation of Ba/F3 cell survival and proliferation. These data demonstrate the existence of several previously undocumented receptor interactions driven by neuregulin.

Deregulated signaling by the four receptor tyrosine kinases encoded by the *erbB* gene family (erbB-1/epidermal growth factor receptor [EGFR], neu/erbB-2/HER2, erbB-3/HER3, and erbB-4/HER4) is implicated in human mammary cancer, ovarian cancer, gastric cancer, and glioblastoma (reviewed in reference 19). Understanding the normal and pathological functions of these receptors requires that their regulation by hormones be elucidated. One complication is that there are at least 15 different agonists for erbB family receptors, including EGF, transforming growth factor α, amphiregulin, betacellulin, heparin-binding EGF-like growth factor, and the several differentially spliced variants of the neuregulins (NRGs), also known as gp30 (27), heregulins (18), neu differentiation factors (35, 54), glial growth factors (28), and acetylcholine receptor-inducing activity (5, 12). Some of these factors bind to and activate signaling by more than one receptor. Moreover, these ligands stimulate nonadditive receptor interactions in cells expressing multiple erbB receptor family members. For example, EGF activates neu when coexpressed with the EGFR, but EGF does not bind or activate neu expressed on its own (22, 50). This transmodulation activation of neu by EGFR apparently works through the formation of EGF-driven receptor heterodimers (15, 53).

NRGs were initially identified as candidate neu ligands by their ability to induce neu tyrosine phosphorylation. The longest forms of NRG contain several different modular domains, including a kringle fold, a C-2 immunoglobulin-like domain, a putative heparan sulfate proteoglycan attachment site, sites for N- and O-linked glycosylation, an EGF homology domain, a hydrophobic membrane-spanning domain, and an intracellular domain of variable length (6, 18, 28, 35, 54). Tissue-specific alternative splicing of NRG transcripts from a single gene results in many NRG isoforms containing different sets of these motifs. Moreover, alternative splicing also produces two types of EGF domain, designated α and β (55). α and β isoforms have different biological activities, which may in part reflect their differential binding affinities to cells expressing receptors for NRG (26).

NRGs are likely to play a significant role in regulating cellular proliferation and differentiation in vivo. NRGs were initially purified from medium conditioned by *ras*-transformed Rat-1 fibroblasts (35) or by the MDA-MB-231 human mammary tumor cell line (18), suggesting that NRGs establish or maintain the growth-transformed phenotype. NRG also affects the proliferation and differentiation of cultured mammary cells. NRG stimulates (18) or inhibits (35, 54) the in vitro proliferation of human mammary tumor cells, which frequently overexpress erbB family receptors (reviewed in reference 19), while NRG stimulates proliferation and milk protein synthesis in a cultured mouse mammary epithelial cell line (29). NRG may also promote wound healing. A single NRG isoform accelerates epidermal migration via increased terminal differentiation of epidermal cells and stimulates integrin expression in the epidermis during wound healing, while wounding stimulates NRG expression in dermal fibroblasts adjacent to the wound (9). NRG also modulates the differentiation and proliferation of neuroectodermal cells. NRGs act as glial cell growth factors (28), may specify a glial cell fate for neural crest stem cells (45), appear to mediate axon-induced mitogenesis of Schwann cells (32), and stimulate acetylcholine receptor synthesis at neuromuscular junctions (5, 12, 20). Furthermore, NRG expression patterns suggest important functions in neurogenesis and in mesenchymal-epithelial cell interactions during development (6, 28, 30, 33).

The physiological responses to agonists for erbB family receptors depend on their ability to coordinately activate multiple receptors that are differentially expressed and have different signaling capabilities. Although NRGs were initially purified by their ability to induce neu tyrosine phosphorylation and were thought to be ligands for neu, NRG does not bind neu and/or induce neu tyrosine phosphorylation in a variety of cell types or in solution (7, 36, 39, 48, 51). Instead, NRG binds erbB-3 (2, 23, 48, 51) and erbB-4 (7, 38, 39, 51). Coexpression of erbB-3 or erbB-4 with neu permits NRG-induced tyrosine

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phosphorylation of neu, presumably through the formation of neu–erbB-3 or neu–erbB-4 heterodimers (2, 23, 39, 48). Despite the many combinatorial possibilities afforded by assortment of four different receptors, interactions of erbB family members with their agonists have been investigated only in a piecemeal fashion. Receptors of different species origins have been mixed in gene transfer experiments, only a subset of receptor combinations have been tested, and interpretation is hampered by the variety of cell backgrounds used and in many cases by the failure to determine the endogenous erbB family receptor expression in the cell lines used. Finally, the hormone-regulated coupling of different erbB family receptors and combinations of receptors to different downstream signaling pathways has not been systematically investigated for any EGF family agonist, including NRGs.

To address these issues, we have undertaken a parallel analysis of the aggregate signaling potential of this receptor family by expressing all four human erbB family receptors, singly and in each pairwise combination, in a uniform cell background. We have used the resulting cell lines for the first comprehensive evaluation of NRG-induced erbB family receptor activation and coupling to cellular responses. The results reveal the pattern of activation of these receptors by NRG and identify novel ligand-induced interactions among these receptors. Moreover, these data suggest several distinct mechanisms by which biological responses are specified by interactions among erbB family receptors and their agonists.

MATERIALS AND METHODS

Cell lines and cell culture. The Ba/F3 mouse pro-B-lymphocyte cell line (34) and its derivatives were grown in RPMI (Gibco/BRL) supplemented with 10% fetal calf serum (Sigma) and interleukin-3 (IL-3) supplied as 10% conditioned medium from the WEHI-3B mouse myelomonocytic leukemia cell line (8). Ba/F3 derivatives transformed with constructs expressing erbB family receptors were grown in medium supplemented with 200 μg of G418 (Gibco/BRL) per ml.

Plasmid constructions. The *Sac*II-*Xho*I fragment of pCO12EGFR (52), which contains the full-length human EGFR cDNA, was subcloned into the *Sma*I site of pBluescript SK−, generating pSKEGFR. The EGFR expression vector pLXSN-EGFR used in the experiments described here was constructed by cloning the 4.2-kb *Xho*I fragment of pSKEGFR, which contains the complete human EGFR cDNA, into the *Xho*I site of the recombinant retroviral expression vector plasmid pLXSN, which carries a neomycin resistance gene under the transcriptional control of the simian virus 40 early promoter (31). The neu expression vector pLXSN-Long-Neu was constructed by cloning the 4.8-kb *Nru*I-to-*Dra*I fragment of pCDNEU (39), which contains the complete human neu cDNA as well as 714 bp of vector sequences 5′ to the neu transcriptional start site, into the *Hpa*I site of pLXSN. Subsequently, the vector sequences 5′ to the neu transcriptional start site were removed by cloning a 4.1-kb *Xho*I fragment of pLXSN-Long-Neu into the *Xho*I site of pLXSN, generating the neu expression vector pLXSN-Neu used in these studies. The erbB-3 expression vector pLXSN-erbB-3 was constructed by cloning the 4.3-kb *Bss*HII fragment of pBSHER3X (40), which contains the complete human erbB-3 cDNA, into the *Hpa*I site of pLXSN. The erbB-4 expression vector pLXSN-erbB-4 was constructed by cloning the 4.6-kb *Sna*BI-to-*Sma*I fragment of pCH4M2 (38), which contains the complete human erbB-4 cDNA, into the *Hpa*I site of pLXSN.

Generation of recombinant Ba/F3 derivatives. Ten micrograms of a single expression vector directing the expression of an erbB family receptor or 5 μg of each of a pair of expression vectors was linearized by digestion with restriction endonucleases and ligated to form concatemers. These were electroporated into 2×10^7 Ba/F3 cells in 0.5 ml of Tris-buffered saline, using a 0.4-cm gap cuvette and a Bio-Rad Gene Pulser set at 200 V and 960 μF. Cells were immediately diluted into 50 ml of culture medium, incubated for 48 h at 37°C, and then seeded in 96-well dishes at 5×10^4 cells per well in medium supplemented with 400 μg of G418 per ml. Drug-resistant lines were expanded and screened for expression of the appropriate erbB family receptor(s). Positive lines were subcloned by limiting dilution and rescreened for receptor expression to ensure homogeneity. The cell lines characterized here are named as follows: LXSN/1 (vector control); EGFR/3; neu/5 and neu/12C; erbB-3/3; erbB-4/7; EGFR + neu/5D; EGFR + erbB-3/4A; EGFR + erbB-4/2A; neu + erbB-3/7A; neu + erbB-4/15A; and erbB-3 + erbB-4/2B.

The ranked order of receptor expression in the double recombinant cell lines is as follows. For EGFR expression, EGFR + erbB-4/2A is higher than EGFR + neu/5D, which is higher than EGFR + erbB-3/4A. For neu expression, neu + erbB-4/15A is marginally higher than EGFR + neu/5D, which is markedly higher than neu + erbB-3/7A. The levels of ErbB-3 expression are similar in the EGFR + erbB-3/4A, neu + erbB-3/7A, and erbB-3 + erbB-4/2B cell lines, while the levels of erbB-4 expression are similar in the EGFR + erbB-4/2A, neu + erbB-4/15A, and erbB-3 + erbB-4/2B cell lines.

Stimulation and analysis of erbB family tyrosine phosphorylation. A total of 2×10^8 recombinant Ba/F3 cells were washed in phosphate-buffered saline (PBS) and resuspended in 50 ml of RPMI supplemented with IL-3. The cells were incubated for 6 h at 37°C, washed in PBS, and resuspended in 1 to 2 ml of PBS. Remaining steps were performed cold or on ice. The cells were transferred in two or three 0.5- to 1.0-ml portions to microcentrifuge tubes. A chemically synthesized NRG β 65-mer peptide (1) corresponding to amino acids 177 to 241 of the NRG β1 isoform (amino acid residues are numbered according to reference 18) or the anti-neu agonistic monoclonal antibody (MAb) TAb 250 (24, 46) was added at a final concentration of 94 ng/ml (NRG) or 10 μg/ml (TAb 250). Control samples remained untreated or were treated with NRG dilution buffer. Following a 10-min incubation, cells were pelleted and incubated for 10 min in 1 ml of EBC lysis buffer (37), which is a Tris-buffered 120 mM sodium chloride solution containing 0.5% Nonidet P-40. Debris was pelleted by centrifugation, and the supernatants were transferred to a fresh tube and diluted 1:3 in EBC to facilitate sample handling. The protein content in each sample was assayed by using Coomassie blue assay reagent (Pierce), and a volume of lysate containing 2 mg of protein was used for each immunoprecipitation.

EGFR was immunoprecipitated with 900 ng of anti-EGFR MAb 528 (14) and 7.2 μg of rabbit anti-mouse antibody 31188 (Pierce); neu was immunoprecipitated with 2 μg of anti-neu MAb TAb 250 (24) and 12 μg of rabbit anti-mouse antibody or with 1 μg of anti-neu MAb FSP-16 (17) and 5 μg of rabbit anti-mouse antibody; erbB-3 was immunoprecipitated with 200 ng of anti-erbB-3 rabbit polyclonal antiserum SC-285 (Santa Cruz Biotechnology); erbB-4 was immunoprecipitated with 1 μg of anti-erbB-4 rabbit polyclonal antiserum SC-283 (Santa Cruz Biotechnology). Specificity of antireceptor antibodies was verified by testing each precipitating antibody for cross-reactivity with cell lines expressing heterologous receptors. All immunoprecipitation mixtures were incubated at 4°C for 2 h, after which the immune complexes were collected by incubation at 4°C with 50 μl of a 10% (vol/vol) suspension of fixed and washed *Staphylococcus aureus* (IGSL-10; The Enzyme Center). Immune complexes were washed three times with NET-N (37) and were eluted from *S. aureus* by boiling in 150 μl of protein sample buffer (37). Samples were divided equally, electrophoresed on separate 7.5% acrylamide–0.17% bisacrylamide–0.1% SDS gels (44), and transferred to nitrocellulose (11) for immunoblotting with either the antiphosphotyrosine MAb 4G10 (Upstate Biotechnology, Inc.) or antibodies specific for receptors. Antibody binding was detected with horseradish peroxidase-coupled sheep anti-mouse antibody NA931 (Amersham) or horseradish peroxidase-coupled donkey anti-rabbit antibody NA934 (Amersham) and enhanced chemiluminescence reagent RPN2106 (Amersham). Immunoblotting antibodies were sheep anti-EGFR polyclonal antibody 06-129 (Upstate Biotechnology Inc.), rabbit anti-sheep antibody 31240 (Pierce), rabbit anti-neu antibody Ab1 (PC04; Oncogene Science), mouse anti-erbB-3 MAb 2F12 (21), and rabbit anti-erbB-4 polyclonal antibody SC-283 (Santa Cruz Biotechnology).

RESULTS

The mouse Ba/F3 pro-B-lymphocyte cell line was chosen for expression of erbB family members because the survival and proliferation of the cells are tightly regulated by exogenous growth factor (IL-3) and because erbB family receptors have not been found to be expressed in mammalian hematopoietic cells. Immunoprecipitation and immunoblotting experiments did not reveal endogenous expression of any erbB family receptors in these cells (42). Nonetheless, we further assessed endogenous receptor expression by PCR amplification of reverse-transcribed transcripts (RT-PCR assay), the most sensitive assay available. RT-PCR analysis of erbB family receptor transcription by using probes homologous to murine erbB family receptor genes in a control Ba/F3 cell line or in Ba/F3 cell lines expressing exogenous human EGFR, neu, erbB-3, or erbB-4 demonstrated that these lines lacked endogenous murine EGFR, neu, or erbB-4 transcription (data not shown). Surprisingly, however, all of the Ba/F3 cell lines tested exhibited detectable levels of endogenous erbB-3 transcription (data not shown). This novel finding implies that erbB family receptors and their ligands may play important roles in the differentiation, expansion, or growth transformation of cells of a B-lymphocyte lineage.

cDNAs directing the expression of erbB family receptors were introduced into Ba/F3 cells to generate clonal lines that

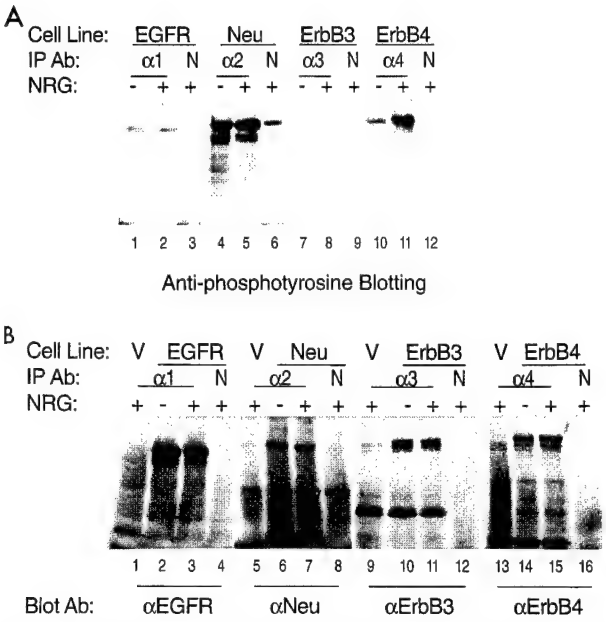

FIG. 1. Regulation of receptor tyrosine phosphorylation by NRG in single recombinant Ba/F3 derivatives. Untreated or NRG-stimulated cell lines were immunoprecipitated with antireceptor antibodies, and portions of immunoprecipitates were analyzed by immunoblotting with antiphosphotyrosine (A) or antireceptor (B) antibodies. V refers to LXSN (vector only) cells. The neu-expressing line used is neu/5. Immunoprecipitating antibodies (IP Ab): α1, anti-EGFR; α2, anti-neu; α3, anti-erbB-3; α4, anti-erbB-4; N, normal mouse or rabbit serum. +, immunoprecipitations of lysates from NRG-treated cells; −, immunoprecipitations of lysates from untreated cells.

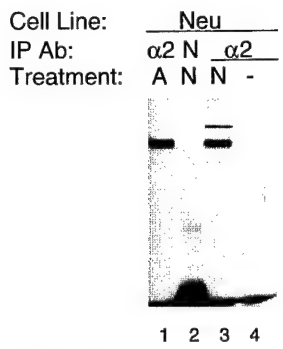

FIG. 2. Regulation of neu/12C cells by NRG. neu/12C cells were incubated with NRG dilution buffer (−), the agonistic anti-neu antibody TAb 250 (46) (A), or NRG (N). The immunoprecipitating antibody (IP Ab) used for immunoprecipitating lysates was anti-neu antibody (α2) or normal mouse serum (N); after immunoprecipitation, lysates were analyzed by immunoblotting with antiphosphotyrosine. The band above neu (lane 3) was not observed in other trials.

express the four different human receptors, singly and in combination. The resulting panel of cell lines was stimulated with a chemically synthesized NRG 65-mer peptide (amino acids 177 to 241 of NRG β1), which encompasses the EGF homology domain and is sufficient for induction of receptor tyrosine phosphorylation (1, 18). Regulation of tyrosine phosphorylation of each receptor by NRG was evaluated by immunoprecipitating the receptors and immunoblotting with antiphosphotyrosine (Fig. 1A and 3A) and antireceptor (Fig. 1B and 3B) antibodies.

Among cell lines expressing a single exogenous receptor (Fig. 1A), NRG failed to stimulate tyrosine phosphorylation of the EGFR (lanes 1 and 2) or erbB-3 (lanes 7 and 8). In contrast, NRG strongly activated tyrosine phosphorylation of erbB-4 (lanes 10 and 11). Since high basal tyrosine phosphorylation of neu in the neu/5 cell line may have obscured the effect of NRG (lanes 4 and 5), we isolated an independent Ba/F3 derivative, designated neu/12C, that expresses considerably less neu than the neu/5 cell line. In this cell line, NRG clearly activated neu tyrosine phosphorylation (Fig. 2, lanes 3 and 4).

In most of the double recombinant cell lines, NRG unambiguously stimulated tyrosine phosphorylation of both erbB family receptors (Fig. 3A; summarized in Table 1). Since the four erbB family receptors have distinct electrophoretic mobilities in most combinations, coprecipitation of heterologous dimerization partners would have been detected. However, coprecipitation was not observed under these conditions. Significantly, the results for the double recombinant cell lines are not simply additive with the responses of single cell lines. For example, NRG does not stimulate tyrosine phosphorylation of the uniquely expressed EGFR, but exogenous coexpression of erbB-3 or erbB-4 with EGFR enabled NRG to regulate EGFR tyrosine phosphorylation. Similarly, while NRG did not stimulate tyrosine phosphorylation of erbB-3 alone, coexpression of EGFR, neu, or erbB-4 permitted activation of erbB-3. Thus, NRG can regulate the tyrosine phosphorylation of each erbB family receptor provided that the appropriate coreceptor is expressed.

While NRG can stimulate the tyrosine phosphorylation of all four erbB family receptors, activation of different receptors or combinations of receptors may specify unique biological responses through coupling of each receptor to distinct cellular signaling pathways. We investigated this possibility by determining whether NRG stimulation enabled survival or growth of the various Ba/F3 derivatives independent of IL-3. Ectopic expression of a number of receptors in Ba/F3 cells permits receptor regulation by the cognate ligands, which in some cases relieves dependence on IL-3 for survival or growth: expression of the erythropoietin receptor with Friend spleen focus-forming virus gp55 permits IL-3-independent proliferation (25). Similarly, EGF stimulation of Ba/F3 cells expressing exogenous EGFR results in EGFR tyrosine phosphorylation and increased cellular DNA synthesis (4, 47), while stimulation of Ba/F3 derivatives expressing exogenous platelet-derived growth factor receptor with platelet-derived growth factor results in receptor tyrosine phosphorylation and IL-3-independent proliferation (43).

In the absence of NRG, all of the Ba/F3 derivatives, even those lines that display substantial basal receptor tyrosine phosphorylation, remained dependent on IL-3 for survival (Table 2). Likewise, NRG stimulation does not confer IL-3-independent survival or growth on control Ba/F3 cells. In cell lines expressing a single exogenous receptor, expression of neu, but not EGFR, erbB-3, or erbB-4, permitted NRG-dependent survival of Ba/F3 cells (Table 2). Indeed, all cell lines engineered to express neu (neu/5, neu/12C [42], EGFR + neu, neu + erbB-3, and neu + erbB-4) survived in the presence of NRG. This survival appears to be dependent on the amount of neu expression, as the neu/5 cell line, which expresses more neu than the other cell lines, also exhibited the strongest IL-3-independent response to NRG, while the neu/12C and neu + erbB-3 cell lines, which express less neu than the other cell lines, exhibited the weakest response to NRG treatment. NRG failed to promote the IL-3-independent survival or proliferation of erbB-4, EGFR + erbB-3, and erbB-3 + erbB-4 lines, even though NRG regulated receptor phosphorylation in these

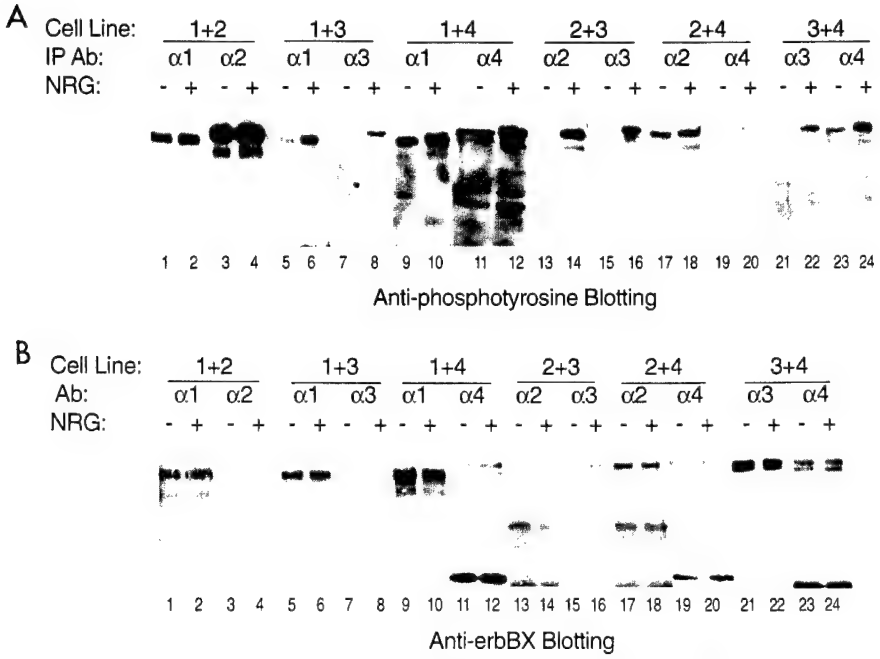

FIG. 3. Regulation of receptor tyrosine phosphorylation in double recombinant Ba/F3 derivatives. Untreated or NRG-treated cells were immunoprecipitated with antireceptor antibodies and analyzed by immunoblotting with antiphosphotyrosine (A) or antireceptor (B) antibodies. Cell lines: 1+2, EGFR + neu; 1+3, EGFR + erbB-3; 1+4, EGFR + erbB-4; 2+3, neu + erbB-3; 2+4, neu + erbB-4; 3+4, erbB-3 + erbB-4. The immunoprecipitating (IP) and/or immunoblotting antibodies (Ab): α1, anti-EGFR antibody; α2, anti-neu antibody; α3, anti-erbB-3 antibody; α4, anti-erbB-4 antibody. +, immunoprecipitations of lysates from NRG-treated cells; −, immunoprecipitations of lysates from untreated cells.

lines (Table 1). Furthermore, while NRG had no effect on the survival of cells expressing either the EGFR or erbB-4 alone, stimulation of EGFR + erbB-4 cells enabled this line to reach saturation densities comparable to those induced by IL-3 (Table 2). For this cell line, NRG acts not as an IL-3-independent survival factor but as a proliferative agent. Hence, not only is NRG-regulated receptor phosphorylation not sufficient for coupling to a cellular response, but the quality of the response is governed by the exact combination of regulated receptors present.

TABLE 1. Summary of NRG-induced erbB family receptor tyrosine phosphorylation and IL-3-independent growth or survival

Cell line	Receptor	NRG-stimulated tyrosine phosphorylation[a]	IL-3-independent growth in the presence of NRG[b]
LXSN		−	N
EGFR		−	S
neu/12C		+	S
erbB-3		−	N
erbB-4		+	N
EGFR + neu	EGFR	+/−	S
	neu	+/−	
EGFR + erbB-3	EGFR	+	N
	erbB-3	+	
EGFR + erbB-4	EGFR	+	P
	erbB-4	+	
neu + erbB-3	neu	+	S
	erbB-3	+	
neu + erbB-4	neu	+/−	S
	erbB-4	+	
erbB-3 + erbB-4	erbB-3	+	N
	erbB-4	+	

[a] Results are abstracted from Fig. 1 to 3 and similar, unpublished data. +, increased receptor tyrosine phosphorylation over basal levels; −, no increase in receptor tyrosine phosphorylation; +/−, borderline results due to high basal levels of receptor tyrosine phosphorylation.

[b] Adapted from Table 2 and similar, unpublished data. N, NRG does not enhance cell survival; S, NRG sustains viable cells; P, NRG induces IL-3-independent proliferation.

DISCUSSION

We have analyzed the NRG-dependent responses conferred upon the Ba/F3 mouse pro-B-lymphocyte cell line by expres-

TABLE 2. Ba/F3 density in response to IL-3 starvation and NRG stimulation[a]

Cell line	Viable cell saturation density (10^3 cells/ml)		
	IL-3 free	IL-3	NRG
LXSN	2	1,765	4
EGFR	1	1,301	3
neu/5	1	2,030	310
erbB-3	<1	1,800	<1
erbB-4	<1	1,583	8
EGFR + neu	2	1,104	162
EGFR + erbB-3	1	1,393	<1
EGFR + erbB-4	3	1,851	1,093
neu + erbB-3	<1	1,258	50
neu + erbB-4	1	1,664	291
erbB-3 + erbB-4	<1	1,475	3

[a] For each trial and treatment, Ba/F3 cells made quiescent by growth to saturation density were plated at a density of 100×10^3/ml in two independently seeded flasks containing medium lacking IL-3 (IL-3 free), medium supplemented with IL-3 (IL-3), or medium lacking IL-3 but supplemented with NRG β 65-mer at a final concentration of 9.4 ng/ml (NRG). Over the next 4 days, cells were stained with trypan blue and counted in a hemacytometer to determine the density of viable cells. For all treatments and cell lines, cells reached viable cell saturation densities with approximately the same kinetics. Data shown are values averaged from two or three independent trials.

sion of the four erbB family receptors, singly and in pairwise combinations. In the presence of appropriate coreceptors, NRG regulated the tyrosine phosphorylation of all four erbB family receptors. While some of the NRG-induced interactions between erbB family receptors observed had been predicted from previous work, we demonstrate here for the first time that in the presence of erbB-3 or erbB-4, NRG regulates tyrosine phosphorylation of the EGFR and that the presence of the EGFR, neu, or erbB-4 enables NRG to regulate tyrosine phosphorylation of erbB3. Furthermore, NRG induces IL-3-independent survival or proliferation in only a subset of the lines that exhibit NRG-induced receptor tyrosine phosphorylation. Thus, the biological responses to NRG are specified at several different levels of regulation.

Previous analyses of NRG-induced signaling by erbB family receptors have been carried out with a few receptor combinations in a variety of cell backgrounds. In mammary cells, NRGs induce neu tyrosine phosphorylation and can be cross-linked to neu, and binding is increased by neu overexpression (36), at first suggesting that neu is a receptor for NRG. However, NRG fails to induce neu tyrosine phosphorylation and/or bind neu when neu is expressed in fibroblasts, ovarian cells (36), CHO cells (7, 39), T-lymphoid cells (39), or COS-7 cells (48), and NRG does not bind to solubilized neu extracellular domains (51). Moreover, NRG binds erbB-3 (2, 23, 48, 51) or erbB-4 (7, 38, 39, 51), and coexpression of erbB-3 or erbB-4 with neu confers NRG responsiveness upon neu, probably through the formation of neu/erbB-3 or neu/erbB-4 heterodimers (2, 23, 39, 48). This finding has led to the general working hypothesis that activation of neu by NRG requires the presence of erbB-3 or erbB-4.

The data presented in this report are compatible with this conclusion and extend the model to include NRG regulation of the EGFR. The EGFR and erbB-3 alone fail to respond to NRG for two different reasons. The EGFR does not bind NRG (18), whereas erbB-3 binds but is impaired for kinase activity (16). The stimulation of tyrosine phosphorylation of neu by NRG might suggest direct activation of neu by NRG but, in view of previously published work, is more likely to reflect interaction with endogenous erbB-3. However, erbB-4 is able to bind and respond to NRG directly.

NRG induces extensive cross talk among receptors expressed in binary combinations (Table 1). Either erbB-3 or erbB-4, both of which bind NRG, enables regulation of the EGFR by NRG. This is the first evidence that NRG can regulate EGFR signaling. As predicted from earlier work, NRG stimulates tyrosine phosphorylation of both receptors in the neu + erbB3, neu + erbB-4, and erbB-3 + erbB-4 cell lines. Coexpression of EGFR, neu, or erbB-4 with erbB-3 permits NRG induction of erbB-3 tyrosine phosphorylation. Although earlier work showed that expression of neu enhances tyrosine phosphorylation and NRG regulation of erbB-3 (2), that work was done in COS-7 cells, which express significant basal amounts of neu and EGFR. Thus, the present work demonstrates for the first time that de novo expression of either the EGFR, neu, or erbB-4 enables hormone-regulated phosphorylation of erbB-3. Endogenous erbB receptor expression in Ba/F3 cells played a limited yet significant role in specifying responses to NRG stimulation in these experiments. While endogenous erbB-3 expression permits NRG stimulation of exogenous neu tyrosine phosphorylation, NRG does not stimulate receptor tyrosine phosphorylation in cells that express exogenous EGFR only. Perhaps the level of endogenous erbB-3 expression in Ba/F3 cells is insufficient to permit NRG-induced EGFR tyrosine phosphorylation. Alternatively, intrinsic differences between the exogenous human and endogenous mouse proteins may result in the different capacities to undergo NRG-induced heterotypic receptor interactions.

While NRG can stimulate the tyrosine phosphorylation of each receptor under the appropriate conditions, the diversity of biological responses to NRG indicates that there must be additional mechanisms by which biological responses to NRG are specified. The patterns of NRG-induced stimulation of erbB receptor tyrosine phosphorylation and IL-3-independent growth and proliferation demonstrate that there are several hierarchical levels at which biological responses to NRG are apparently specified.

First, responsiveness to NRG requires the expression of erbB-3 or erbB-4. Previous work and results presented here establish that NRG cannot bind or stimulate tyrosine phosphorylation of erbB family receptors in the absence of erbB-3 or erbB-4 expression. Biological responses to NRG are also specified by the intrinsic kinase activity of the erbB family receptor(s) stimulated by NRG, since the kinase-deficient erbB-3 requires the presence of a coreceptor for hormone-regulated phosphorylation.

Distinct biological responses to NRG are also conferred by ligand-induced coupling of different erbB family receptors to different signaling pathways. NRG enables the IL-3-independent survival of cell lines expressing neu, probably through NRG-induced activation of neu via erbB-3. However, NRG does not enable the IL-3-independent survival of EGFR + erbB-3 cells or of erbB-4 cells, even though NRG stimulates receptor tyrosine phosphorylation in these lines. This finding demonstrates that neu has signaling properties distinct from those of the EGFR, erbB-3, or erbB-4, consistent with earlier work showing that different erbB family receptors can activate different signaling pathways and responses (3, 10, 13, 21, 41, 49). Neither NRG nor EGF induces IL-3-independent proliferation of cells that individually express erbB-4 or EGFR (Table 2 and reference 42), yet NRG stimulates IL-3-independent proliferation in the EGFR + erbB-4 cell line. One simple explanation would be that IL-3-independent proliferation requires activation of two independent pathways, one activated by the EGFR and one activated by erbB-4. An interesting alternative would be that EGFR and erbB-4 phosphorylation sites are different in ligand-induced EGFR/erbB-4 heterodimers than in ligand-induced receptor homodimers owing to substrate specificity of the receptor catalytic domains and steric considerations in the cross-phosphorylation reaction. This would permit recruitment of unique signaling proteins to the heterodimer, resulting in unique biological responses. Thus, the diversity of hormone-regulated outputs from this receptor outwork may extend beyond the simple combinatorial possibilities.

Since EGF and NRG bind to different receptors, it can be predicted that individual members of the EGF family of ligands activate different constellations of erbB family receptors, so that these different ligands will yield distinct patterns of biological responses. Preliminary evidence supports this prediction. Three different members of the EGF family induce different patterns of erbB family receptor tyrosine phosphorylation and IL-3-independent growth in the Ba/F3 derivatives described here (42). Given the multitude of roles that erbB family receptors and their ligands apparently play in diverse biological processes such as neurogenesis, neuromuscular signaling, tumorigenesis, wound healing, and the regulation of mesenchymal-epithelial cell interactions, it is likely that all of the mechanisms described here play a significant part in specifying responses to ligand stimulation in vivo.

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Heregulin Induces *in Vivo* Proliferation and Differentiation of Mammary Epithelium into Secretory Lobuloalveoli[1]

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Abstract

Mammary gland development and differentiation is mediated through the combined activities of systemic hormones and locally synthesized growth factors. To determine the *in vivo* response of mammary epithelium to heregulin (HRG), we implanted Elvax pellets containing HRGα or HRGβ within the mammary glands of prepubescent female mice in the presence or absence of exogenous estradiol and progesterone (E/P). Mice treated in the same way with transforming growth factor α (TGF-α) were included as a positive control. Each growth factor treatment induced epithelial ductal branching in the presence or absence of E/P. In the absence of E/P, HRGβ did not affect terminal end bud formation, mammary epithelium branching, or ductal migration. In contrast, TGF-α and HRGα induced ductal branching and HRGα induced ductal migration in the absence of E/P. The overall mammary response to growth factors was potentiated by the concomitant presence of E/P. In every case, the *in vivo* mammary epithelial responses to HRGα were more robust than TGF-α. Limited lobuloalveolar development was also observed in growth factor-treated mammary glands when E/P was present. Histological examination of growth factor-induced lobuloalveoli revealed secretory products within the lumen of HRGα and HRGβ lobuloalveoli. TGF-α-induced lobuloalveoli lacked similar secretory products.

Introduction

Mammary gland development is unusual in that the vast majority of growth and differentiation occurs postnatally. In the prepubescent mouse, mammary ductal structures emanating from the nipple terminate in large bulbous structures referred to as TEBs.[3] With the onset of puberty, steroid hormones function as potent mitogens of TEB. This rapidly dividing cell population is responsible for ductal growth and branching during expansion of the mammary gland. During pregnancy, an additional pronounced growth cycle results in increased ductal branching and lobuloalveolar development. The lobuloalveoli terminally differentiate into milk-producing structures, and the extensive lobuloalveoli completely fill the interductal spaces during lactation (1, 2). These developmental processes are regulated through a complex series of events requiring the activities of both intraglandular and systemic hormones/growth factors (3–5). The steroid hormones estrogen and progesterone are major players in these developmental processes. However, the exact mechanisms underlying steroid hormone growth effects are not known and may involve a combination of direct effects and/or stimulation of growth factors which in turn mediate mammary gland development in a juxtacrine or autocrine fashion. Indeed, estradiol stimulates mammary epithelial expression and/or secretion of several EGF family members (6–10), and these growth factors have several important functional roles during mammary gland development (4, 5, 11).

Normal breast tissue expresses several EGF family members including EGF (12), TGF-α (12–16), amphiregulin (16–18), cripto-1 (16–18), and HRG (19). In addition, mammary gland expression of all four EGFR family members identified to date (*e.g.*, EGFR, erbB-2/HER-2/neu, erbB-3, and erbB-4) has been reported (19–22). A substantial body of evidence suggests that the EGF family of growth factors and their cellular receptors play an important role in both normal and malignant mammary gland development (4, 5, 11, 23–27). Most recently, the function of HRG in mammary gland development has been investigated. In mammary tumor cells, HRGs appear to have a mitogenic effect (28–31) or induce differentiation of mammary epithelium with the synthesis of milk proteins (30, 32–34). Yang *et al.* (19) examined the affects of HRG on mammary gland morphogenesis. In whole-organ culture, HRG stimulates lobuloalveolar development and the production of milk proteins. A putative role for HRG in lobuloalveolar development and milk production is further supported by the following observations; HRGα is expressed within the mammary mesenchyme adjacent to lobuloalveolar structures, and HRGα expression is regulated during mammary gland development and is only expressed during pregnancy (19). Therefore, HRG appears to be a po-

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[3] The abbreviations used are: TEB, terminal end bud; EGF, epidermal growth factor; EGFR, EGF receptor; HRG, heregulin; TGF-α, transforming growth factor α; E/P, estradiol and progesterone; RP-HPLC, reverse phase high pressure liquid chromatography.

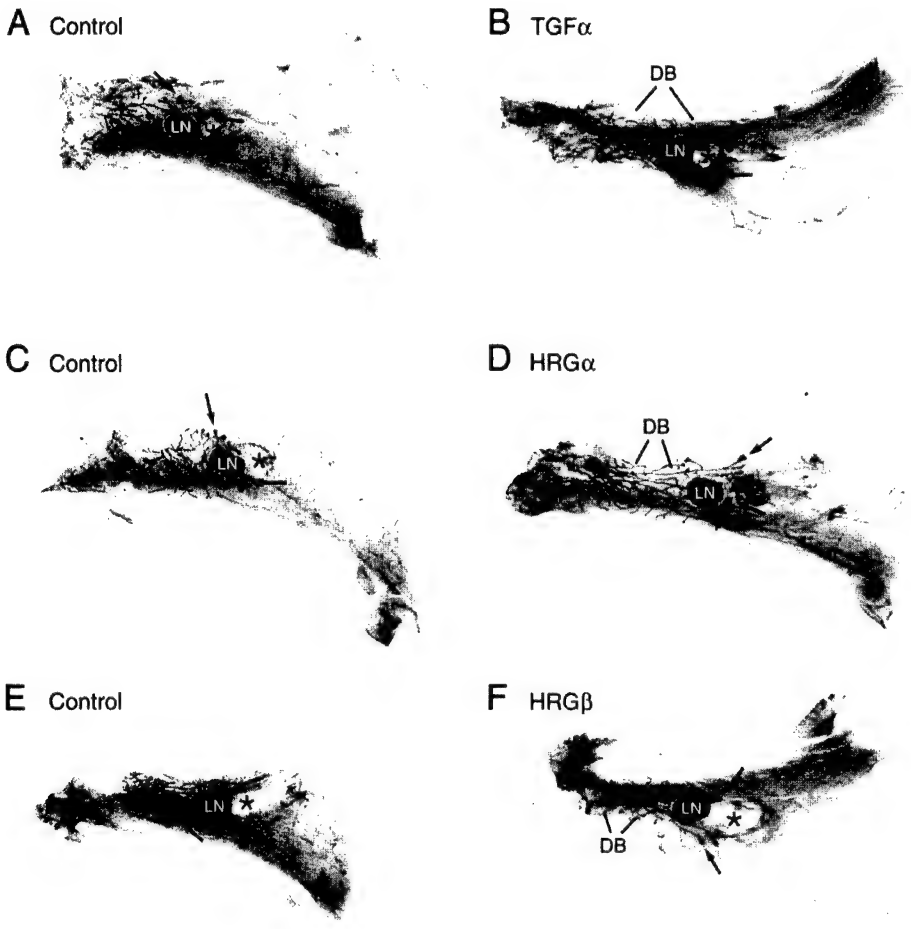

Fig. 1. Effect of growth factor treatment on mammary gland morphology in the absence of estradiol and progesterone. Control Elvax pellets and pellets containing growth factor were implanted within the surgically exposed number 4 inguinal mammary fat pad of 30-day-old female BALB/c mice. Implants were positioned (*) immediately anterior to the central lymph node (*LN*). Mice were sacrificed 3 days following implant insertion, and excised mammary glands were processed using the whole-mount procedure and stained with carmine (see "Materials and Methods"). Terminal end buds are indicated by *arrows*, and regions of growth factor-induced ductal branching (*DB*) are indicated. Implants contained 10 μg of TGF-α (*B*), 5 μg of HRGα (*D*), and 10 μg of HRGβ (*F*). Contralateral control for each sample is represented (*A*, *C*, and *E*).

tent and developmentally important mammary epithelial growth factor.

Despite these recent observations, the role of HRG in mammary gland morphogenesis *in vivo* has not been investigated. To address this issue, we have used a mouse model system to examine the direct effects of HRG on mammary epithelium under conditions similar to those where this growth factor normally functions. The mammary glands of prepubescent female mice were surgically implanted with Elvax pellets containing recombinant HRGα, HRGβ, or TGF-α in the presence or absence of steroid hormones. All three growth factors possessed differing levels of epithelial mitogenic activity *in vivo*. In addition, in the presence of steroid hormones, each growth factor induced epithelial differentiation into lobuloalveolar structures. However, only the HRG-treated lobuloalveoli underwent terminal differentiation, resulting in the lumenal accumulation of secretory products. Taken together, these experiments offer the first *in vivo* evidence for a role of HRG in mammary epithelial development and terminal differentiation into milk protein-secreting lobuloalveolar structures.

Results

HRG Induces Ductal Branching *in Situ*. HRG induces pleiotropic responses in cultured mammary epithelial cells (19, 28, 29, 31, 33–35); however, the *in vivo* response of mammary epithelium to this family of growth factors has not been investigated. As a first step toward identifying a biological role for HRG in mammary ductal morphogenesis, we surgically implanted slow-release pellets containing varying amounts of HRGα or HRGβ within the developing mammary fat pad of virgin female mice. HRGα and HRGβ are splice variants that possess differing EGF domains (36). Pellets lacking growth factor were inserted into the contralateral fat pad as a negative control. Previously, TGF-α has been shown to induce ductal branching and lobuloalveolar development in a similar experimental system (37) and was, therefore, included as a positive control in our experiments. The mice were sacrificed 3 days after implant insertion, and whole mounts of the mammary glands were examined for ductal morphogenesis and lobuloalveolar development.

When compared to contralateral controls, each growth factor induced ductal branching within the treated mammary gland (Figs. 1 and 2). Responses to growth factors in the

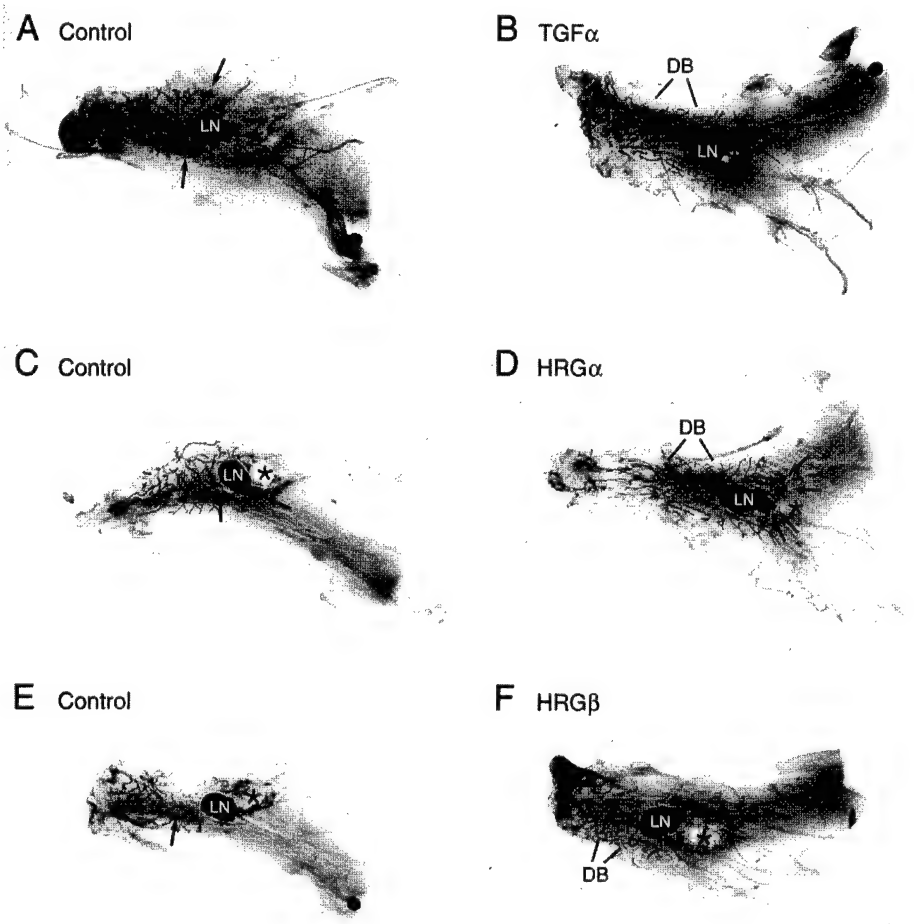

Fig. 2. Effect of growth factor treatment on mammary gland morphology in the presence of estradiol and progesterone. Control Elvax pellets containing 10 μg of 17 β-estradiol and 1 mg of progesterone (E/P) and pellets containing growth factor with E/P were implanted within the surgically exposed number 4 inguinal mammary fat pad of 30-day-old female BALB/c mice. Implants were positioned (*) immediately anterior to the central lymph node (*LN*). Mice were sacrificed 3 days following implant insertion, and excised mammary glands were processed using the whole-mount procedure and stained with carmine (see "Materials and Methods"). Terminal end buds are indicated by *arrows*, and regions of growth factor-induced ductal branching (*DB*) are indicated. Implants contained 10 μg of TGF-α (*B*), 5 μg of HRGα (*D*), and 10 μg of HRGβ (*F*). Paired contralateral control for each sample is represented (*A, C,* and *E*).

presence or absence of E/P were maximal with pellets containing 10 μg of TGF-α or HRGβ or 5 μg of HRGα (data not shown). These growth factor concentrations were used in all subsequent experiments. In the absence of E/P, each growth factor induced ductal branching in the region posterior to the central lymph node (Fig. 1, compare *panels A* to *B*, *C* to *D*, and *E* to *F*). However, differences between each growth factor could be identified. For example, the ductal branching observed in HRGβ-treated glands (Fig. 1*F*) was less extensive than glands treated with either TGF-α (Fig. 1*B*) or HRGα (Fig. 1*D*). In addition, where TGF-α and HRGβ treatment appeared to inhibit TEB formation (Fig. 1, *B* and *F*, respectively), HRGα not only induced TEB proliferation but also increased ductal migration anterior to the central lymph node (Fig. 1*D*).

Treatment of control mammary glands with E/P alone resulted in a slight increase in ductal diameter (compare Fig. 1*A* to Fig. 2*A*). Moreover, the mammary response to growth factors was potentiated by the presence of E/P because ductal branching induced by each growth factor was more pronounced in the presence of E/P (compare Fig. 1, *B, D,* and *F,* to Fig. 2, *B, D,* and *F,* respectively). The mammary epithelial responses to implants containing TGF-α (Fig. 2*B*) and HRGβ (Fig. 2*F*) were similar because both growth factors inhibited TEB formation. In contrast, HRGα induced TEB formation, and the overall epithelial response to HRGα was more robust (Fig. 2*D*) than either TGF-α (Fig. 2*B*) or HRGβ (Fig. 2*F*).

The extent of ductal branching, ductal growth, and TEB formation induced by each growth factor in the presence and absence of E/P was quantitated. Data from 10 mice, for each experimental condition, was subjected to statistical analysis. Due to high variability among mice, each quantitated parameter was normalized to the contralateral control within an individual animal. Although each growth factor induced ductal branching (Figs. 1 and 2), branching induced by HRGβ was statistically significant only in the presence of E/P (Fig. 3). In general, HRGα appeared to induce a more robust and pleiotropic response within treated mammary glands than either TGF-α or HRGβ. Indeed, ductal branching was more extensive in HRGα-treated glands whether in the presence or absence of E/P (Fig. 3). Moreover, whereas TGF-α and HRGβ appeared to slightly inhibit or had no affect on TEB formation, HRGα induced TEB proliferation in the presence of E/P (Fig. 3). Moreover, HRGα was the only growth factor to significantly increase ductal length within treated mammary glands

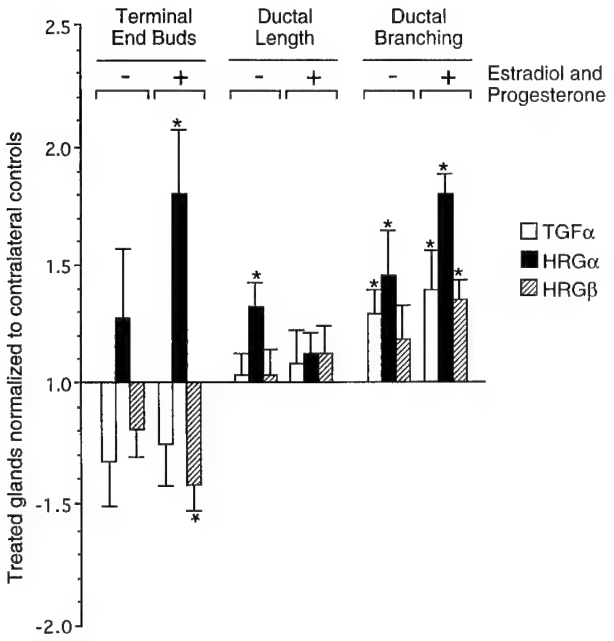

Fig. 3. Effects of growth factors on mammary epithelium. Ten 30-day-old female BALB/c mice were implanted with growth factor pellets at the growth factor's concentration of maximal response in the absence (−) or presence (+) of 10 μg of estradiol and 1 mg of progesterone. Mice were sacrificed 3 days following implant insertion, and excised mammary glands were processed using the whole-mount procedure and stained with carmine (see "Materials and Methods"). The number of TEBs, the ductal length of the longest duct per fat pad, and the number of ductal branches was determined for each treated and contralateral control gland. Treated glands were normalized to their contralateral controls, and the means plus 1 SD (*bars*) are represented above. Growth factor-induced phenotypes significantly different from contralateral controls (paired student *t* test; $P < 0.01$) are indicated (*). Implants contained 10 μg of TGF-α, 5 μg of HRGα, and 10 μg of HRGβ.

(Fig. 3). With the exception of increased ductal length induced by HRGα in the absence of E/P, the concomitant presence of E/P appeared to potentiate the mammary epithelial response to each growth factor tested (Fig. 3).

HRG Induces Mammary Lobuloalveolar Development and the Accumulation of Lumenal Secretory Products *in Vivo*. Whole mounts prepared from growth factor-treated mammary glands revealed extensive epithelial ductal branching. We next examined the terminal ducts within treated and control mammary glands for evidence of lobuloalveolar structures. As expected, untreated glands or glands treated with E/P alone did not develop lobuloalveolar structures. In contrast, a limited extent of lobuloalveolar development was observed in a majority of mammary glands treated with growth factor (Fig. 4, *A-C*). Growth factor-induced lobuloalveoli required the concomitant presence of E/P because similar structures were not observed in glands treated with growth factors alone. Consistent with previous results, the effect of HRGα was more robust than either TGF-α or HRGβ. HRGα induced lobuloalveolar development in 77% of treated mammary glands, whereas TGF-α and HRGβ induced lobuloalveoli in 39 and 46% of treated glands, respectively (13 glands were examined for each treatment). Histological examination of growth factor-induced lobuloalveoli revealed numerous epithelial buds typical of these structures (Fig. 4, *D-F, arrows*). In addition, HRGα- and HRGβ-induced lobuloalveoli exhibited accumulation of lumenal secretory products (Fig. 4, *E* and *F*), which stained positive for β-casein by immunohistochemistry (data not shown). Similar accumulations were not observed in TGF-α-induced lobuloalveoli (Fig. 4*D*).

Discussion

Mammary gland development involves a complex and highly regulated sequence of postnatal events. Recently, expression of an EGF-related subfamily of growth factors termed the neu differentiation factors or HRGs was detected *in vivo* within connective tissue juxtaposed to fully differentiated, milk-secreting lobuloalveoli (19). To determine if HRG plays a role in mammary epithelial growth and/or differentiation *in vivo*, we inserted slow-release pellets containing HRG within mammary glands of prepubescent mice and analyzed the *in vivo* response of mammary epithelium to these growth factors. We found that HRGα and HRGβ induced epithelial branching and differentiation into lobuloalveolar structures, as does a related growth factor, TGF-α. However, histological examination of TGF-α- and HRG-induced lobuloalveoli revealed a striking difference; HRGα and HRGβ stimulated the accumulation of lumenal secretory products, including the milk protein β-casein, within treated lobuloalveoli. TGF-α-induced lobuloalveoli lacked similar lumenal accumulations. These results suggest that HRG can induce terminal differentiation of mammary epithelium *in vivo* into milk protein-secreting lobuloalveolar structures.

The epithelial response to growth factor implants was potentiated by the concomitant presence of estradiol and progesterone. Indeed, lobuloalveoli were only observed in the presence of these steroid hormones. A similar requirement of estradiol and progesterone for EGF- and HRG-induced lobuloalveoli in mammary organ culture has been reported (4, 19, 38). Some evidence suggests that the requirement of exogenous steroid hormones may also reflect strain differences. For example, TGF-α implants induce lobuloalveoli development in CH3/HeN mice in the absence of exogenous estradiol and progesterone (37). In contrast, our results indicate that induction of lobuloalveoli by TGF-α or HRG in BALB/c mice requires the concomitant presence of exogenous estradiol and progesterone. Although we did not perform experiments to determine if estradiol or progesterone alone could augment mammary responses to growth factors, substantial evidence indicates that both estradiol and progesterone have independent proliferative effects on mammary epithelium. Furthermore, co-administration of these hormones enhances independent proliferative effects (3). Using mice carrying a null mutation in the progesterone receptor, Lydon *et al.* (39) demonstrated the *in vivo* requirement of progesterone in ductal epithelium proliferation and lobuloalveoli differentiation. Thus, it seems probable that progesterone contributes to the growth factor-induced lobuloalveolar development observed in our experiments. Although estradiol is considered to be the steroid hormone most directly involved in mammary epithelial proliferation (3), the exact role of estradiol in mammary development is poorly defined. The

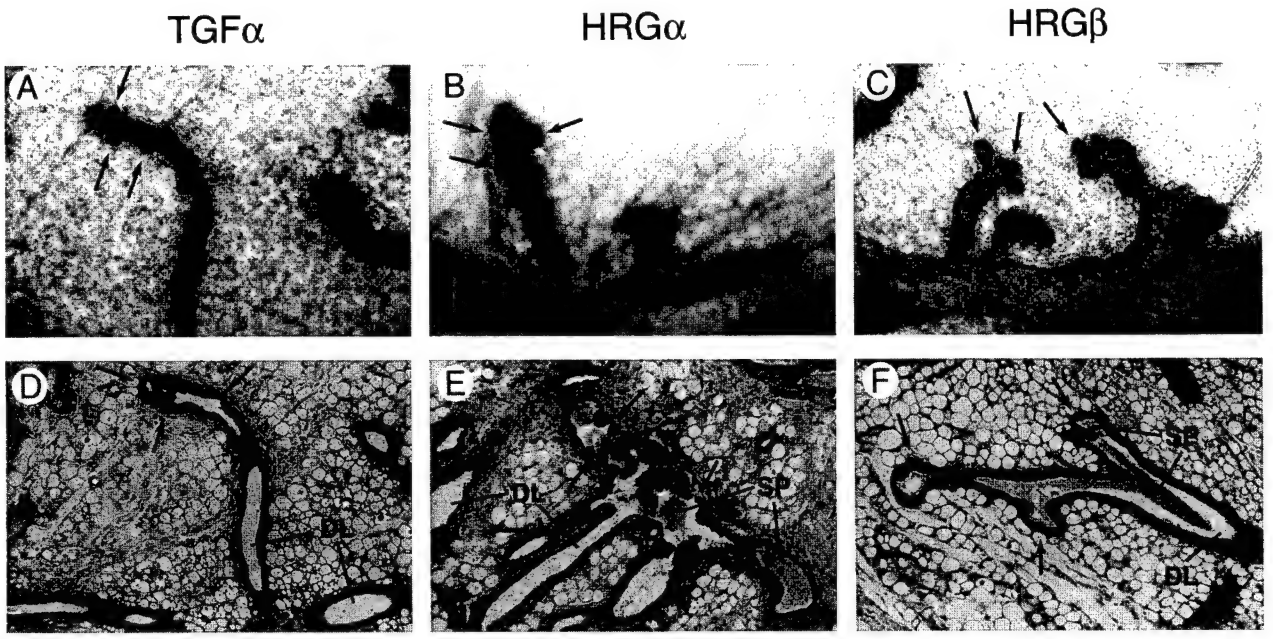

Fig. 4. Growth factor-induced lobuloalveolar development in prepubescent female mice. Elvax pellets containing growth factor with 10 μg of 17 β-estradiol and 1 mg of progesterone were implanted within the surgically exposed number 4 inguinal mammary fat pad of 30-day-old female BALB/c mice. Mice were sacrificed 3 days following implant insertion, and excised mammary glands were processed using the whole-mount procedure and stained with hematoxylin (see "Materials and Methods"). Photomicrographs of paraffin sections revealed lobuloalveolar structures in growth factor-treated glands (*A-C*). *Arrows* indicate epithelial buds typical of these structures. Histological examination of paraffin-embedded lobuloalveoli stained with H&E (*D* and *E*) revealed secretory products (*SP*) within the ductal lumens (*DL*) of HRGα- and HRGβ-treated mammary glands (*E* and *F*, respectively). Implants contained 10 μg of TGF-α (*A* and *D*), 5 μg of HRGα (*B* and *E*), and 10 μg of HRGβ (*C* and *F*).

reason for this lack of clarity lies in the fact that in addition to independent proliferative effects, estradiol also induces mammary expression of several growth factors, including the EGF family members EGF (7), TGF-α (6, 7, 40), and amphiregulin (9, 41). Mammary gland expression of these EGF family members may directly affect mammary development and thereby augment the epithelial response to growth factor implants observed in our experiments.

The qualitative and quantitative differences in mammary epithelial responses to TGF-α and HRG can be explained through functional differences of the two growth factors. Histochemical analysis of mouse mammary glands reported elsewhere revealed dramatic differences in the cellular localization and expression of these growth factors. Mammary gland expression of TGF-α was detected during each epithelial developmental stage with the exception of lactation, and immunostaining within the cap cell layer of the TEB and epithelial cells of subtending ducts was observed (12). In contrast, expression of HRG is induced during pregnancy within the connective tissue adjacent to ductal and lobuloalveolar structures (19). Differing mammary epithelial responses to TGF-α and HRG may also reflect the activation of different signaling tyrosine receptor kinases within these cellular populations. TGF-α binds directly to the EGFR (26) and can activate erbB-2 (42), erbB-3, and erbB-4,[4] presumably through a ligand-driven receptor cross-phosphorylation mechanism (43, 44) also referred to as "transmodulation" (24, 45). Similarly, HRG binds directly to erbB-3 (46, 47) and erbB-4 (47) and can drive the activation of EGFR and erbB-2 (46–48). Evidence from *in vitro* experiments indicates that cellular responses to signaling by this family of receptors can be radically different, depending upon both the transmodulation partner and the activating growth factor (48–51). Therefore, one prediction follows that signaling by EGFR family members *in vivo* would also induce a diversity of cellular responses that are dependent upon the activating growth factor. Cellular responses to HRG *in vivo* appear to be regulated primarily but not exclusively through erbB-2 signaling. Disruption of HRG or erbB-2 in transgenic mice results in a similar embryonic lethal phenotype characterized by nearly identical heart malformations and neural crest development defects (52, 53). Moreover, expression patterns within the developing rhombencephalon suggest that a HRG: erbB-2 autocrine or paracrine signaling relationship has been disrupted in these mice (52, 53). These observations further support a direct relationship between HRG and erbB-2 signaling. A similar relationship may mediate HRG activity in mammary epithelium, and we are presently designing experiments to examine this possibility.

In our experiments, the *in vivo* response of mammary epithelium to HRGα was more robust than HRGβ. This result was surprising because *in vitro* experiments consistently identify HRGβ as the more potent growth factor (28, 30, 31, 33). However, we used chemically synthesized and bacterial recombinant peptides in our experiments, which may not

[4] D. J. Riese II, E. Kim, G. Allison, S. Buckley, M. Klagsbrun, G. D. Plowman, and D. F. Stern. J. Biol. Chem., in press.

represent the complete activities of full-length HRG protein. Alternatively, the enhanced mammary response to HRGα may reflect a physiological role for HRGα and not HRGβ in mammary gland development. Indeed, only HRGα isoforms are expressed in the mammary gland, and this expression is induced during pregnancy (19). Thus, the HRGα expression pattern strongly correlates with the *in vivo* function identified in this communication. Our experiments provide the first demonstration of an important *in vivo* role for HRG in mammary epithelium proliferation and differentiation into secretory lobuloalveoli. In conclusion, we propose that HRGα is the physiologically relevant HRG isoform expressed within the developing mammary gland, and HRGα plays an important role in the differentiation of mammary epithelium into milk-secreting lobuloalveoli.

Materials and Methods

Plasmid Construction. The human HRG β1 cDNA fragment corresponding to residues 177–244 (54) was subcloned into the pNB261 bacterial expression vector as follows. Poly(A) mRNA was isolated from cultured human MDAMB231 cells (American Type Culture Collection) by use of the Fast Track mRNA isolation kit (InVitrogen), according to the manufacturer's instructions. The HRG β1 cDNA fragment corresponding to residues 177–244 was amplified by a 30-cycle reverse transcription-PCR procedure using the RNA Gene Amp kit (Perkin-Elmer Corp.) and the primers incorporating 5′ *Eco*RI (sense 5′-CGCGAATTCTATGAGCCATCT-TGTAAAATGTGC) and *Hin*dIII (anti-sense 5′-CGCGAAGCTTAGTA-CAGCTCCTCCGCCTCCAT) linkers. The 204-bp amplified fragment was digested with *Eco*RI/*Hin*dIII and inserted into the same sites of the Bluescript vector pCRII (Stratagene). The nucleotide sequence of the 204-bp insert was confirmed by use of an Applied Biosystems Automated Sequencer using standard methods. The sequenced 204-bp insert was excised from pCRII by digestion with *Eco*RI/*Hin*dIII and subcloned downstream of the trp-inducible promoter using the same restriction sites of the pNB261 expression vector (construct pHerβ1ST). The sequence of the 204-bp human HRG β1 insert was confirmed as described above.

Expression and Purification of Human Recombinant HRG β1 (177–244). For large scale fermentation and expression of HRG β1 (177–244), pHERβ1ST was transformed into the *Escherichia coli* strain GE81. Bacterial cells from a 10-liter fermentation in modified M9 medium (55) were harvested by centrifugation, and expression was induced by resuspending the bacteria pellet into fresh modified M9 medium lacking tryptophan. After an induction period of 4 h, a total of 69 g of cell paste was recovered by centrifugation. Expression of the predicted 7000-Da product peaked at 3 h postinduction. A 25-g bacterial pellet was resuspended into 50 ml of lysis buffer [20 mM Tris (pH 8.0), 40 mM NaCl, 0.25 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride] and passed through a French press twice. The lysate was centrifuged for 45 min at 12,000 rpm, and the pellet was resuspended into 20 ml of lysis buffer. Fifteen mg of hen egg white lysozyme (Sigma Chemical Co.) was added, and the mixture was incubated at room temperature for 20 min. Three hundred μl of 1 mg/ml DNase and 800 μl of 100 mM $MgCl_2$ were added, and the mixture was incubated at room temperature for an additional 15 min. The suspension was centrifuged for 15 min at 12,000 rpm, and the final inclusion body pellet was washed twice with 1.0% NP40 and once with ddH_2O and lyophilized to dryness to yield 500 mg of dried inclusion bodies. A suspension of 10 mg of inclusion bodies in 2.0 ml of 50 mM Tris (pH 6.0), 6 M guanidinium hydrochloride, and 200 mM DTT was heated at 37°C for 2 h and filtered through a Costar 3.5-μm spin-filter; then the filtrate was diluted into 100 ml of folding buffer [50 mM Tris (pH 8.6), 1 M urea, 1.5 mM glutathione, 0.75 mM glutathiol, and 10 mM methionine] and stirred for 5 days at 4°C. The folded, oxidized protein was isolated by RP-HPLC on a VYDAC C-4 reverse phase column using an acetonitrile/ddH_2O/0.1% trifluoroacetic acid gradient. The isolated protein was homogeneous by RP-HPLC and capillary electrophoresis, and was composed of 206 μg by amino acid analysis. The protein exhibited a mass of 7877.8 Da by electrospray mass spectrometry [theoretical mass for oxidized HRG β1 (177–244) is 7878.1 Da].

Growth Factors. HRGα 177–228 (HRGα 52) was synthesized on an Applied Biosystems 430A peptide synthesizer using standard *tert*-butyloxycarbonyl chemistry protocols provided by the manufacturer (version 1.40; *N*-methylpyrolidone/hydroxybenzotriazole). Peptide was purified by RP-HPLC, characterized by electrospray mass spectroscopy, and analyzed for disulfide bonding as described previously (56). Peptide quantities were determined by amino acid analysis. Human recombinant TGF-α was purchased from Collaborative Biomedical Products.

Implant Preparation. Growth factor peptides and steroid hormones were encapsulated within Elvax pellets essentially as described elsewhere (57). Briefly, a lyophilized mixture containing growth factor peptide and, where indicated, the steroid hormones 17-β-estradiol (10 μg; Sigma) and progesterone (1 mg; Sigma) was suspended in 25 μl of Elvax (generously donated by Elf Atochem, Philadelphia, PA) dissolved previously in dichloromethane (15% w/v). The entire suspension was transferred to an Eppendorf tube, snap-frozen in liquid nitrogen, and dried under vacuum. The dried Elvax pellet was compressed between tweezers such that the final pellet was ~1 mm in diameter and weighed 2–3 mg.

Surgical Implantation. Thirty-day-old virgin female BALB/c mice (Charles River) were used in all experiments. Mice were anethesized with an i.p. injection of 250–350 μl of avertin [20 mg/ml 2,2,2-tribromoethanol (Aldrich) in saline]. The number 4 inguinal mammary fat pad was surgically exposed, and a 2-mm incision was made through the mammary fat pad outer membrane immediately anterior to the central lymph node. The Elvax pellet was placed within the incision and immobilized under the mammary fat pad outer membrane. Control pellets lacking growth factor were inserted into the contralateral number 4 inguinal mammary fat pad. The wounds were closed using surgical staples, and the mice were allowed to recover under a heat lamp.

To determine the response range and saturation point for each growth factor, mice were implanted with Elvax pellets containing 0.5, 1.0, 2.0, 5.0, 10, or 20 μg of growth factor. In another series of experiments, pellets contained 10 μg of 17 β-estradiol and 1 mg of progesterone (E/P) in addition to growth factor.

Whole-Mount Preparation of Mammary Gland. Mice were sacrificed 3 days following placement of implants. The entire number 4 inguinal mammary fat pad was removed at the nipple and spread onto a precleaned glass slide. The fat pad was air-dried for 10 min and fixed in acidic ethanol (75% ethanol and 25% acetic acid) for 1 h at room temperature. The tissue was incubated in 70% ethanol for 15 min and ddH_2O for 5 min. Ductal structures were stained in carmine solution [0.2% carmine and 0.5% aluminum potassium sulfate (both from Sigma)] for 12–16 h at room temperature. The stained tissue was dehydrated through graded ethanol, defatted in acetone, and cleared in toluene for 12–16 h. The stained and cleared mammary fat pad was mounted under coverslip with Permount (Fisher) and photographed with a slide duplicator.

Histological Examination. For histological examination of mammary gland ductal structures, fat pads were fixed in 4% paraformaldehyde, stained in hematoxylin or carmine solution, dehydrated through graded ethanol into xylene, and cleared in methyl salicylate (Sigma). Ductal structures identified under a dissecting microscope were excised, blocked in paraffin, sectioned at 4 μm, and stained with H&E using standard procedures.

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Betacellulin activates the epidermal growth factor receptor and erbB-4, and induces cellular response patterns distinct from those stimulated by epidermal growth factor or neuregulin-β

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Betacellulin is a member of the epidermal growth factor (EGF) family. These soluble proteins are ligands for one or more of the four receptor tyrosine kinases encoded by the erbB gene family (erbB-1/epidermal growth factor receptor (EGFR), neu/erbB-2/HER2, erbB-3/HER3 and erbB-4/HER4). While evidence suggests that betacellulin is a ligand for the EGFR, the ability of betacellulin to regulate other erbB family receptors has not been analysed. Previously we engineered derivatives of the mouse Ba/F3 hematopoietic cell line to ectopically express erbB family receptors, singly and in pairwise combinations. We have stimulated this panel of cell lines with betacellulin and two other EGF family members, EGF itself and neuregulin-β (NRG-β). In the cell lines expressing a single erbB family receptor, betacellulin not only stimulated EGFR tyrosine phosphorylation, but it activated erbB-4 as well. Furthermore, in the double recombinant Ba/F3 derivatives, betacellulin stimulated a complex pattern of receptor phosphorylation distinct from the patterns activated by NRG-β and EGF. Moreover, betacellulin stimulated a complex pattern of interleukin-3 independence in the Ba/F3 derivatives distinct from those activated by NRG-β and EGF. These data identify a novel receptor for betacellulin and establish that different EGF family ligands activate distinct patterns of receptor phosphorylation and coupling to cellular signaling pathways.

Keywords: betacellulin; EGF; neuregulin; EGF receptor; ErbB receptors; signaling

Introduction

The epidermal growth factor (EGF) family consists of at least 15 members, including epidermal growth factor, transforming growth factor alpha (TGFα), amphiregulin (AR), betacellulin, heparin-binding-EGF-like growth factor (HB-EGF), cripto, epiregulin and the several differentially-spliced variants of neuregulin (NRGs), also known as heregulins, neu differentiation factors, gp30, glial growth factors and acetylcholine receptor inducing activity (Reviewed in Groenen, *et al.*, 1994). These factors activate a family of four receptor tyrosine kinases encoded by the erbB gene family (erbB-1/epidermal growth factor receptor (EGFR), neu/erbB2/HER2, erbB-3/HER3, and erbB-4/HER4). The patterns of receptor activation stimulated by EGF family ligands is very complex; some of these peptides bind to and activate signaling by more than one erbB family receptor. Furthermore, in cell lines expressing multiple erbB receptor family members, these ligands can activate erbB family receptors that do not bind these ligands when the receptors are expressed individually, a process called transmodulation (Reviewed in Hynes and Stern, 1994; Earp *et al.*, 1995). For example, EGF activates neu when coexpressed with the EGFR, but EGF does not bind or activate neu expressed on its own (Akiyama, *et al.*, 1988; King *et al.*, 1988; Stern and Kamps, 1988; Connelly and Stern, 1990). It has been proposed that transmodulation occurs through the formation of EGF-driven receptor heterodimers and receptor cross-phosphorylation (Goldman *et al.*, 1990; Wada *et al.*, 1990; Qian *et al.*, 1992; Spivak-Kroizman *et al.*, 1992). Not only does EGF induce erbB receptor transmodulation, but every EGF family member tested thus far has this activity (Stern and Kamps, 1988; Johnson *et al.*, 1993; Plowman *et al.*, 1993; Carraway *et al.*, 1994; Kita *et al.*, 1994; Sliwkowski *et al.*, 1994; Karunagaran *et al.*, 1995; Riese *et al.*, 1995).

Betacellulin was initially identified as a factor in the conditioned medium of a mouse pancreatic β cell carcinoma (insulinoma) cell line that is mitogenic for Balb/C 3T3 cells. The 80 amino acid mature betacellulin protein is derived from a 177 amino acid membrane-bound precursor and contains the six conserved cysteine residues that are arranged in a characteristic pattern common to all of the EGF family members. Furthermore, betacellulin has significant overall homology to mature EGF, TGF-α, AR, HB-EGF, and NRGs (Sasada *et al.*, 1993; Shing *et al.*, 1993; Reviewed in Groenen *et al.*, 1994). Binding of human recombinant betacellulin to the A431 human adenocarcinoma cell line, which overexpresses the EGFR, can be competed with an excess of EGF, suggesting that betacellulin is a ligand for the EGFR (Watanabe *et al.*, 1994). However, previous studies have not assessed betacellulin binding to other erbB family receptors or betacellulin stimulation of erbB family receptor tyrosine phosphorylation or coupling to cellular signaling pathways.

Little is known about the *in vivo* functions of betacellulin. While its expression in the BTC-3 mouse insulinoma cell line (Shing *et al.*, 1993) and the MCF-7 human breast adenocarcinoma cell line (Sasada *et al.*, 1993) implies that betacellulin regulates the prolifera-

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tion of pancreatic and breast cells and may play a causative role in breast and pancreatic cancer, the receptor(s) for betacellulin must be identified before definitive studies of the physiologic effects of betacellulin can be undertaken. In this study we have used derivatives of the Ba/F3 mouse pro-B lymphocyte cell line that have been engineered to ectopically express, singly and in pairwise combinations, all four erbB family receptors (Riese *et al.*, 1995). We have assessed erbB family receptor tyrosine phosphorylation and the biological responses of these cell lines to stimulation with betacellulin and two other EGF family ligands, neuregulin-β (NRG-β) and EGF itself. Not only do these experiments identify a novel receptor for betacellulin, but we also demonstrate that betacellulin stimulates complex patterns of erbB family receptor tyrosine phosphorylation and coupling to cellular signaling pathways that are unique from the patterns stimulated by EGF and NRG-β.

Results

erbB family receptor tyrosine phosphorylation in single recombinant cell lines

The Ba/F3 cell line is a mouse pro-B lymphocyte cell line (Palacios and Steinmetz, 1985) that does not express endogenous EGFR, neu and erbB-4, but does express low levels of erbB-3 (Riese *et al.*, 1995). We have previously described the generation of Ba/F3 derivatives that ectopically express the four human erbB family receptors, singly and in all pairwise combinations (Riese *et al.*, 1995). In this report, we have stimulated this panel of cell lines with recombinant human betacellulin, NRG-β and EGF and assessed erbB family receptor tyrosine phosphorylation. The recombinant human betacellulin used in these experiments consisted of 34 amino acids of the human amphiregulin precursor (Val107–Arg140), linked to the 50 amino acid EGF-structural motif of human betacellulin (Arg31–Tyr80) and a 9 amino acid hemagglutinin epitope. The amphiregulin sequences in this molecule are not within the EGF-structural motif and therefore are not predicted to contribute to receptor binding. Furthermore, this recombinant betacellulin produced as a tagged fusion construct in bacteria had an activity indistinguishable from that produced transiently in COS cells using an expression construct containing the full length human betacellulin sequence (Plowman *et al.*, in preparation).

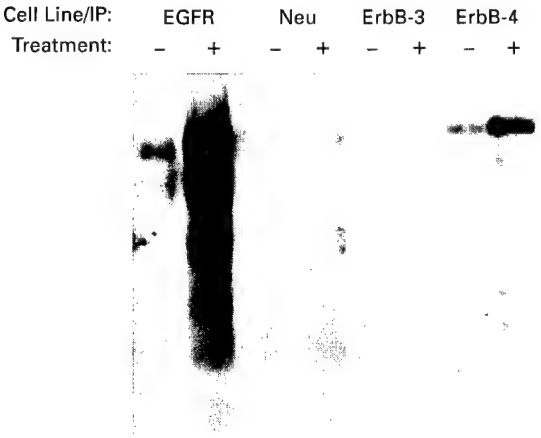

Figure 1 Regulation of receptor tyrosine phosphorylation by betacellulin in single recombinant Ba/F3 derivatives. Lysates from untreated or betacellulin-treated Ba/F3 derivatives were immunoprecipitated with anti-receptor antibodies and analysed by immunoblotting with anti-phosphotyrosine antibody. The cell lines and immunoprecipitating antibodies are as marked. Lysates from betacellulin-treated cells are denoted '+' while lysates from untreated cells are denoted '–'

In cell lines that ectopically express a single erbB family receptor, both betacellulin and EGF stimulated EGFR tyrosine phosphorylation (Figures 1 and 2a). Surprisingly, betacellulin (as well as NRG-β) stimulated erbB-4 tyrosine phosphorylation (Figures 1 and 2a). Not only do these data demonstrate that betacellulin regulates EGFR signaling, but they also indicate that erbB-4 is a receptor for betacellulin. Betacellulin did not stimulate increased neu or erbB-3 tyrosine phosphorylation (Figures 1 & 2a), suggesting that neither neu nor erbB-3 is a receptor for betacellulin (NRG-β stimulation of neu tyrosine phosphorylation is probably due to endogenous erbB-3 expression (Riese, *et al.*, 1995). However, because erbB-3 has only minimal intrinsic tyrosine kinase activity (Guy *et al.*, 1994), these data do not rule out the possibility that erbB-3 can bind betacellulin.

Since some AR sequences were present in the recombinant betacellulin used in these experiments, there was a remote possibility that these contributed to the unanticipated activation of erbB-4. Hence, we compared the activities of our betacellulin preparation with those of a recombinant mature-form preparation comprised of betacellulin exclusively (R and D Systems). In the cell line expressing erbB-4, both betacellulin preparations stimulated similar maximal levels of erbB-4 tyrosine phosphorylation at 3 nM betacellulin (data not shown). This maximal level was similar to that stimulated by 6 nM NRG-β (data not shown). Therefore, stimulation of EGFR and erbB-4 phosphorylation by betacellulin was not conferred by AR sequences.

erbB family receptor tyrosine phosphorylation in double recombinant cell lines

In activating both the EGFR and erbB-4, betacellulin displays activities distinct from EGF, which activates the EGFR alone, and NRG-β, which binds to erbB-3 and erbB-4. We next compared the effects of betacellulin, EGF, and NRG-β on receptor transmodulation by assessing receptor tyrosine phosphorylation in cell lines expressing combinations of erbB family receptors. Betacellulin, as well as EGF, stimulated the tyrosine phosphorylation of both receptors in the EGFR + neu (1+2), EGFR + erbB-3 (1+3), and EGFR + erbB-4 (1+4) cell lines (Figure 2b and c). Therefore, both betacellulin and EGF can transmodulate the other three receptors when co-expressed with EGFR. However, the three ligands did not stimulate equal levels of receptor phosphorylation. In 1+3 cells, all three ligands stimulated approximately equal levels of erbB-3 phosphorylation, while betacellulin and EGF stimulated higher levels of EGFR phosphorylation than NRG-β did (Figure 2b). Similarly, in 1+4 cells,

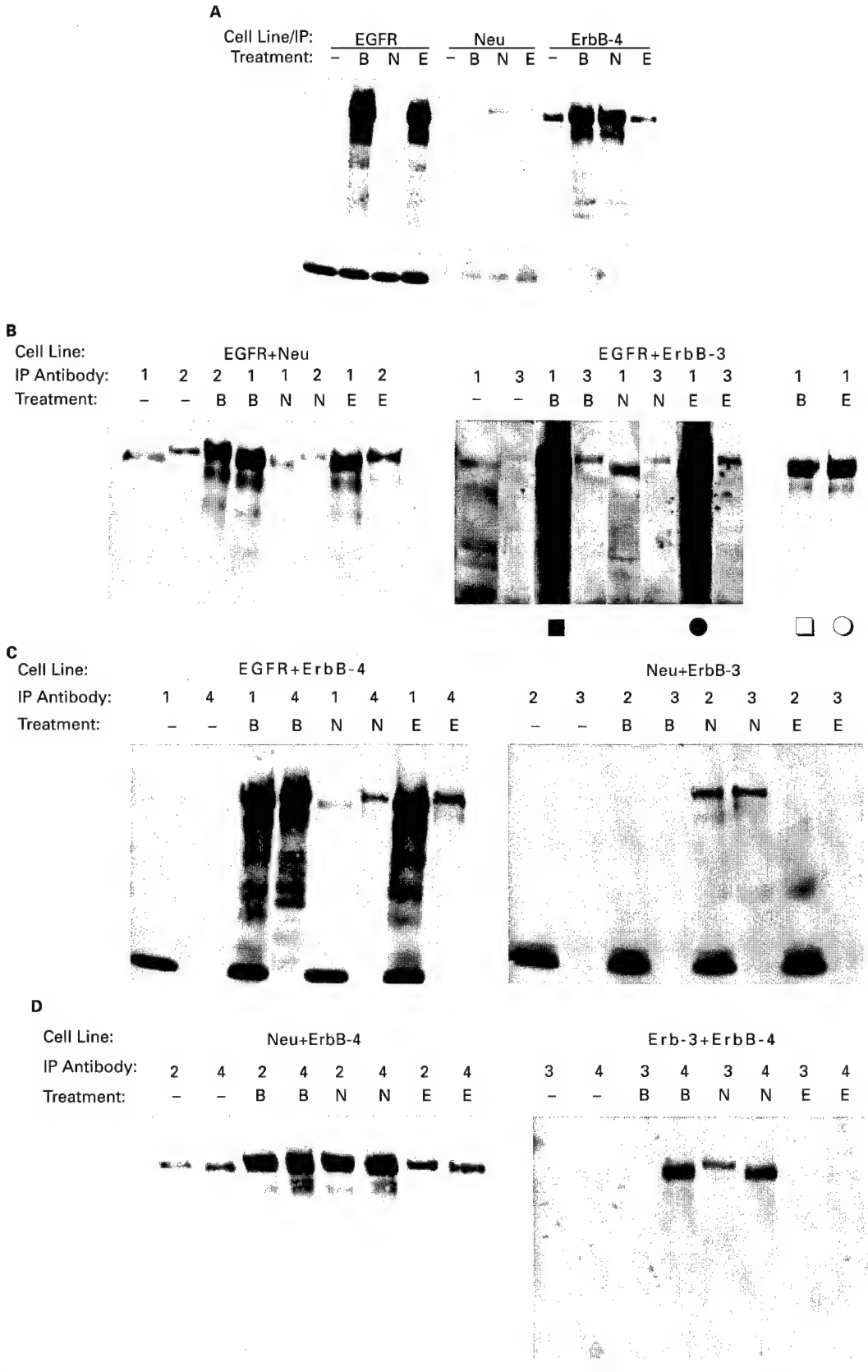

Figure 2 (**A**–**D**) Comparison of receptor tyrosine phosphorylation in Ba/F3 derivatives stimulated with betacellulin, EGF, or NRG-β. Lysates from untreated or ligand-treated Ba/F3 derivatives were immunoprecipitated with anti-receptor antibodies and analysed by immunoblotting with anti-phosphotyrosine antibody. The cell lines are as marked. Immunoprecipitating antibodies were: 1, anti-EGFR; 2, anti-Neu; 3, anti-erbB-3; 4, anti-erbB-4. Lysates from betacellulin-treated cells are denoted 'B', lysates from NRG-β-treated cells are denoted 'N', lysates from EGF-treated cells are denoted 'E' and lysates from untreated cells are denoted '–'. Lighter exposures of the lanes denoted by ■ and ● are denoted by □ and ○

betacellulin stimulated approximately equal levels of EGFR and erbB-4 phosphorylation, while EGF stimulated higher levels of EGFR phosphorylation than erbB-4 phosphorylation and NRG-β stimulated higher levels of erbB-4 phosphorylation than EGFR phosphorylation (Figure 2c). One explanation for these differences is that EGFR, erbB-3, and erbB-4 may have a lower affinity for heterotypic interactions and transmodulation than for the homotypic interactions induced by direct stimulation.

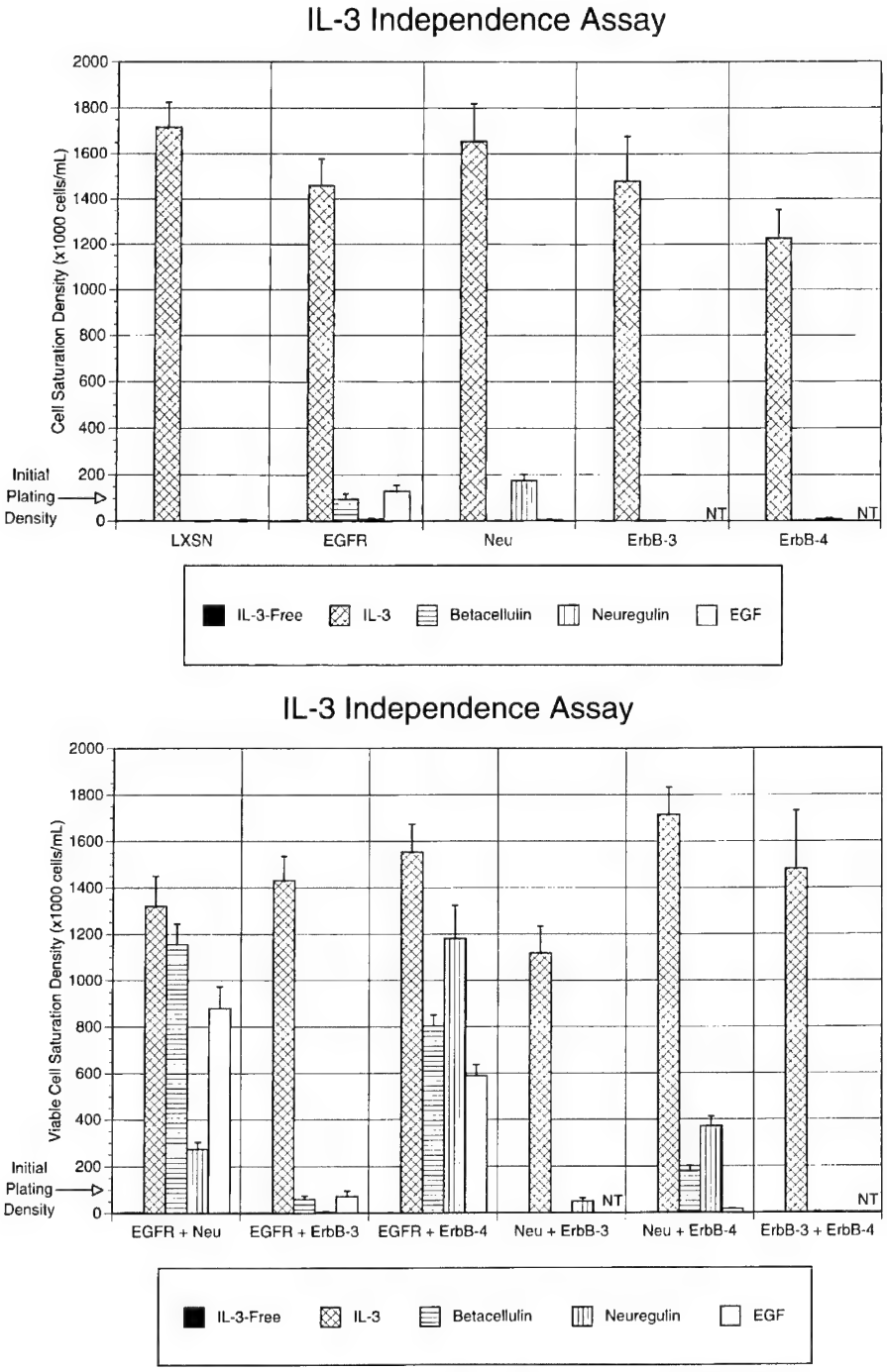

Figure 3 IL-3-independent saturation density of Ba/F3 cells treated with betacellulin, EGF, or NRG-β. Ba/F3 derivatives were plated at a density of 100×10^3 cells/ml in medium lacking IL-3 (IL-3-Free), medium supplemented with IL-3 (IL-3), or in medium lacking IL-3 but supplemented with 10 ng/ml human recombinant EGF (EGF), 9.4 ng/ml NRG-β 65- mer (Neuregulin), or 7 ng/ml human recombinant betacellulin (Betacellulin). Viable cell saturation densities were calculated 2 to 4 days after seeding. Each combination of cell lines and factors was tested 4 to 20 times and the arithmetic means of the viable cell saturation densities are indicated by the filled bars, while the standard error of the means are indicated by the error bars. Because cultures treated with IL3-free medium exhibited densities of less than 4×10^3 cells/ml, these values, represented by the left-most bar for each cell line, are not apparent on the graphs. Some of the data describing the responses to stimulation with NRG-β or control media has been previously published (Riese *et al.*, 1995). 'NT' indicates not tested

As expected from the response of the erbB-4 cell line, betacellulin and NRG-β stimulated erbB-4 tyrosine phosphorylation in the three double recombinant cell lines that express erbB-4. However, while both betacellulin and NRG-β stimulated EGFR and neu tyrosine phosphorylation in the EGFR + erbB-4 (1+4) and neu + erbB-4 (2+4) cell lines, respectively, betacellulin did not stimulate erbB-3 tyrosine phosphorylation in the erbB-3 + erbB-4 (3+4) cell line, even through NRG-β did (Figures 2c and d). Finally, betacellulin did not stimulate phosphorylation of either receptor in neu + erbB-3 cells, while NRG-β did (Figure 2c), suggesting that neither neu nor erbB-3 is a receptor for betacellulin.

IL-3-independent growth of Bal/F3 derivatives

We next tested whether betacellulin, NRG-β, and EGF stimulate different patterns of receptor coupling to cellular signaling pathways. Since the recombinant Ba/F3 cell lines are strictly dependent upon interleukin-3 (IL-3) for survival and proliferation (Riese *et al.*, 1995), we tested whether betacellulin, NRG-β, or EGF induced the IL-3-independent survival or proliferation of the various Ba/F3 derivatives. Activation of either EGFR or neu in the single recombinant cell lines was associated with IL-3 independent survival but not proliferation (Figure 3a), while activation of erbB-3 or erbB-4 in the single recombinants had no biological effect (Figure 3a). Therefore, ligand stimulation of erbB phosphorylation was necessary, but not sufficient, for an IL-3 independent response (Table 1).

We also assessed ligand activity in the double recombinant Ba/F3 cell lines (Figure 3b). As expected from the responses of the single recombinant cell lines, receptor activation in cells expressing EGFR or neu conferred, with one notable exception, a minimal response of IL-3 independent survival. For example, in the EGFR + neu (1+2), EGFR + erbB-4 (1+4), neu + erbB-3 (2+3), and neu + erbB-4 (2+4) cell lines, receptor activation stimulated a minimum of IL-3-independent survival, while in erbB-3 + erbB-4 (3+4) cells none of the ligands stimulated an IL-3 independent response. The exception is the response of EGFR + erbB-3 (1+3) cells to ligand stimulation. As predicted, betacellulin and EGF stimulated the IL-3 independent survival of 1+3 cells; however, NRG-β failed to stimulate an IL-3 independent response, despite stimulating both EGFR and erbB-3 tyrosine phosphorylation in this cell line (Figure 2b).

Furthermore, in some of the double recombinant cell lines ligand stimulation of coupling of multiple receptors to signaling pathways acted in a nonadditive manner to stimulate an IL-3 independent response (Figure 3b). In 1+2 cells betacellulin and EGF stimulated IL-3 independent proliferation, while in 1+4 cells betacellulin and NRG-β stimulated IL-3 independent proliferation and EGF stimulated a response intermediate to survival and proliferation. Therefore, while activation of either EGFR alone or neu alone stimulated IL-3 independent survival, in some cases activation of EGFR along with either neu or erbB-4 conferred IL-3 independent proliferation.

Discussion

Previous reports demonstrated that betacellulin binds to the A431 human adenocarcinoma cell line, which overexpresses the EGFR. This binding was quenched by the addition of an excess of EGF, suggesting that betacellulin is a ligand for the EGFR (Watanabe *et al.*, 1994). However, the effect of betacellulin on EGFR tyrosine phosphorylation and signaling and the possibility that betacellulin might activate other erbB family receptors were not assessed. Here we show that in Ba/F3 cells expressing only a single ectopic erbB family receptor, betacellulin stimulates the tyrosine phosphorylation of both the EGFR and surprisingly, erbB-4 (Table 1). This is consistent with the

Table 1 Summary of stimulation of receptor tyrosine phosphorylation and IL-3 independence

Cell line	*Receptor*	*Betacellulin tyr phos.*[a]	*Betacellulin IL-3 indpt.*[b]	*NRG-β tyr phos.*[a]	*NRG-β IL-3 indpt.*[b]	*EGF tyr phos.*[a]	*EGF IL-3 indpt.*[b]
EGFR		+	S	–	N	+	S
Neu		–	N	+[c]	S[c]	–	N
erbB-3		–	N	–	N	–	NT[d]
erbB-4		+	N	+	N	–	NT[d]
EGFR+Neu	EGFR	+	P	*[c]	S[c]	+	P
	Neu	+		*[c]		+	
EGFR+erbB-3	EGFR	+	S	+	N	+	S
	erbB-3	+		+		+	
EGFR+erbB-4	EGFR	+	P	+	P	+	S/P
	erbB-4	+		+		+	
Neu+erbB-3	Neu	–	N	+	S	–	NT[d]
	erbB-3	–		+		–	
Neu+erbB-4	Neu	+	S	+	S	–	N
	erbB-4	+		+		–	
erbB-3+erbB-4	erbB-3	–	N	+	N	–	N
	erbB-4	+		+		–	

[a] Results are abstracted from Figures 1 and 2, Riese *et al.*, 1995, and similar unpublished data. '+' indicates increased receptor tyrosine phosphorylation over basal levels, '–' indicates no increase in receptor tyrosine phosphorylation, and '*' indicates ambiguity due to high basal levels of receptor phosphorylation. [b] Results are abstracted from Figure 3. 'N' indicates no IL-3 independent response, 'S' indicates stimulation of IL-3 independent survival, 'P' indicates stimulation of IL-3 independent proliferation, and 'S/P' indicates stimulation of an intermediate response. 'NT' indicates not tested. [c] The response to NRG is apparently due to interactions with the endogenous erbB-3 in Ba/F3 cells (Riese *et al.*, 1995). [d] Given the absence of receptor tyrosine phosphorylation, no IL-3 independent response is expected

observation that radiolabeled betacellulin binds specifically to EGFR and erbB-4, but not to neu (Plowman *et al.*, in preparation). Thus, betacellulin exhibits activities that are distinct from those displayed by EGF, which activates the EGFR alone, and NRG-β, which activates erbB-3 and erbB-4 (Table 1). Furthermore, in this first comprehensive analysis of erbB family transmodulation for both betacellulin and EGF, we find that EGF can transmodulate erbB-4 in the EGFR + erbB-4 cell line (Table 1). We also demonstrate that betacellulin stimulates a pattern of receptor transmodulation that is qualitatively distinct from the patterns stimulated by EGF and NRG-β.

With one exception, betacellulin, EGF, and NRG-β transmodulated the tyrosine phosphorylation of all four erbB family receptors in cell lines that express any receptor for each ligand (Table 1). Thus, differences in ligand activities in the double recombinant cell lines can be predicted by differences in activities in the single recombinant cell lines. For example, NRG-β activates erbB-3, while betacellulin does not activate neu or erbB-3. Not surprisingly, in cells expressing neu + erbB-3, NRG-β stimulates the phosphorylation of both receptors, while betacellulin does not stimulate the phosphorylation of either receptor in this cell line (Table 1). The single exception is the response of the erbB-3 + erbB-4 (3+4) cell line to betacellulin. Both betacellulin and NRG-β stimulate erbB-4 tyrosine phosphorylation in the single recombinant cell line. However, in 3+4 cells, NRG-β stimulates the trosine phosphorylation of both receptors, while betacellulin stimulates the tyrosine phosphorylation of erbB-4 but not of erbB-3 (Table 1). Nonetheless, because NRG-β binds erbB-3, it is possible that this absence of erbB-3 tyrosine phosphorylation may not be due to differences between betacellulin- or NRG-β-induced erbB-3 transmodulation.

Previous work demonstrated that different erbB family receptors or combinations of receptors couple to distinct cellular signaling pathways. For example, EGFR activation stimulates the tyrosine phosphorylation of four proteins that are not highly phosphorylated following neu activation (Fazioli *et al.*, 1992). Furthermore, activated erbB-3 stimulated higher levels of phosphatidylinositol 3-kinase than EGFR did (Fedi *et al.*, 1994; Soltoff *et al.*, 1994; Carraway *et al.*, 1995) and it has been suggested that EGFR and neu bind the adapter protein GRB2, but erbB-3 does not (Prigent and Gullick, 1994; but also see Kim *et al.*, 1994; Fedi *et al.*, 1994). These different coupling capacities of the erbB family receptors can be correlated to specific biological responses. Activation of the EGFR stimulates the IL3 independent proliferation of 32D myeloid cells, while wild-type and mutationally-activated neu alleles do not (DiFiore *et al.*, 1990). In Ba/F3 cells, however, activation of neu stimulates IL-3 independent survival, while activation of EGFR and erbB-4 together stimulates IL-3 independent proliferation (Riese *et al.*, 1995).

We found that betacellulin stimulates IL-3 independent survival or proliferation in neu + erbB-4 cells and in every cell line that expresses EGFR. In contrast, EGF stimulated IL-3 independence only in those cell lines that express EGFR while NRG-β stimulated IL-3 independence only in the EGFR + erbB-4 cell line and in those cell lines that express neu (Table 1). Therefore, with a single exception, the minimal requirement for IL-3 independence is activation of either EGFR or neu. The exception is that betacellulin and EGF, but not NRG-β, stimulated IL-3 independent survival in the EGFR + erbB-3 cell line (Table 1). This lack of response to NRG-β may merely reflect the lower level of EGFR phosphorylation stimulated by NRG-β in this cell line (Figure 2b). On the other hand, the absence of biological response may reflect the different mechanism by which the EGFR is activated. We demonstrated previously that coupling of multiple receptor species to cellular signaling pathways acts in a non-additive manner in specifying biological responses (Riese *et al.*, 1995). Accordingly, while activation of EGFR or neu by themselves stimulated IL-3 independent survival, activation of EGFR and either neu or erbB-4 together stimulated IL-3 independent proliferation (Table 1).

Biological responses to EGF family ligands are regulated by several hierarchical mechanisms (Riese *et al.*, 1995). Some, but not all, of these mechanisms are shared by other networks of receptor tyrosine kinases and their ligands, including the neurotrophin network and the fibroblast growth factor (FGF) network. Like the EGF family, the neurotrophin and FGF ligand families have several members that can each activate multiple receptors. The neurotrophin ligand family includes nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4, also known as NT-4/5 or NT-5 (Reviewed in Barbacid, 1994), while the FGF family has at least 9 members encoded by different genes (Reviewed in Johnson and Williams, 1993). Furthermore, like the erbB receptor family, both the FGF receptor and neurotrophin receptor families have multiple members (FGFR-1, FGFR-1, FGFR-3, FGFR-4 and TrkA, TrkB, TrkC, respectively). Moreover, like the EGFR and erbB-4, some of these FGFRsand Trks can bind multiple ligands (Reviewed in Johnson and Williams, 1993; Barbacid, 1994).

Another regulatory mechanism common to the EGF/erbB and FGF signaling networks is that both use heparan sulfate proteoglycans (HSPGs) to modulate receptor-ligand interactions. FGFs bind with low affinity in a multivalent manner to HSPGs, causing ligand oligomerization (Reviewed in Lemmon and Schlessinger, 1994) and increasing their binding affinity for FGFRs (Reviewed in Eckenstein, 1994). Because the FGF/FGFR complex exists in a 1:1 stoichiometry (Spivak-Kroizman *et al.*, 1994), yet FGFs are monomeric, it has been proposed that HSPG binding potentiates FGF stimulation of FGFR phosphorylation and dimerization. HSPGs also regulate the interactions of EGF family ligands with their receptors. Several EGF family ligands bind HSPGs, including NRGs, amphiregulin (AR), and heparin-binding-EGF-like growth factor (HB-EGF), and this binding regulates ligand-receptor interactions (Aviezer and Yayon, 1994; Johnson and Wong, 1994; Cook, *et al.*, 1995a,b). However, many of the mechanistic details of regulation by HSPGs have yet to be elucidated.

While the neurotrophin and FGF networks have regulatory mechanisms that are also features of the EGF/erbB network, there are also features of the neurotrophin and FGF networks that are not properties of the EGF/erbB network. Alternative splicing

produces truncated FGFR and Trk isoforms lacking the cytoplasmic tyrosine kinase domain and sites for tyrosine phosphorylation (Reviewed in Johnson and Williams, 1993; Barbacid, 1994). Therefore, a regulatory mechanism not observed in the EGF/erbB network results in dominant negative receptors, which are not a characteristic of the EGF/erbB network. Another feature that is characteristic of the neurotrophin network and not seen in the EGF/erbB network is regulation by a low-affinity co-receptor. p75, the low-affinity neurotrophin receptor, has no tyrosine kinase domain (Reviewed in Chao, 1994) and p75 binding is in some cases dispensable for biological response (Reviewed in Ibanez, 1994). Nonetheless, it has been proposed that p75 regulates the biological response to neurotrophins by altering the affinity of neurotrophin binding to the Trk family receptors (Benedetti *et al.*, 1993; Reviewed in Chao, 1994).

Data presented here suggests that differences in NRG-β, EGF and betacellulin activities play a significant role in specifying the proliferation and differentiation of human tissues *in vivo*. These ligands may play their most significant role in the mammary epithelium and tissues of neuroectodermal origin. Not only has the expression of EGF family ligands been documented in these cell types, but these ligands can regulate the proliferation and/or differentiation of these cell types in cultured cell or animal model systems. Furthermore, mounting evidence suggests that increases in the expression and/or signaling of erbB family receptors plays a significant role in tumors of mammary or neuroectodermal origin (Reviewed in Hynes and Stern, 1994). Because betacellulin, NRG-β and EGF have distinct biological activities that apparently reflect their differing abilities to activate receptor signaling, it may be possible to develop antagonists that specifically disrupt signaling by a single EGF family ligand and may inhibit the genesis or growth of malignancies without disrupting the activity of other EGF family ligands in the same tissue.

Materials and methods

Production of human recombinant Betacellulin

To facilitate refolding and purification, human recombinant betacellulin was produced as an epitope-tagged fusion with human amphiregulin. The pPL-Lambda (Pharmacia) thermoinducible bacterial expression vector was modified to remove the EcoRI, BamHI and SmaI sites upstream from the PL promoter and a human betacellulin transcription unit was inserted into the unique HpaI site within the N gene. The insertion contained the lac and Cro gene Shine-Delgarno ribosome binding sites; a unique BglII cloning site; an initiating methionine codon; the nucleotide sequence encoding 34 amino acids of the human amphiregulin precursor (Val107–Arg140), the 50 amino acid EGF-structural motif of human betacellulin (Arg31–Tyr80) and a 9 amino acid hemagglutinin epitope sequence (PYDVPDYAS); a stop codon; unique EcoRV and XbaI restriction sites; and transcription termination sequences. The resulting plasmid, pPLABTC-Tag, was transformed into competent *E. Coli* N4830-1 and grown at 30°C in 1 litre LB media with 50 ug/ml ampicillin to an Abs_{600} of 0.7. Cultures were then induced by incubation at 42°C for 18–24 h. Following induction, cells were harvested by centrifugation at 5000 *g*, washed in STE buffer (50 mM Tris, pH 8.0/200 mM NaCl/2 mM EDTA). The pellet was resuspended in STE containing 2 mM 2-mercaptoethanol, and lysed by addition of 0.2 mg/ml lysozyme followed by addition of Triton X-100 and Zwittergent (CalBiochem) to 1%. To ensure lysis and solubilization of non-inclusion body protein, the preparation was sonicated for 2 min and centrifuged at 13 000 *g* for 10 min. The pellet was resuspended in STE and sonicated again for an additional 1 min. The slurry was then layered on a 40% sucrose cushion and centrifuged at 13 000 *g* for 10 min at 4°C. The inclusion body pellet was resuspended in 6 M guanidine-HCl (GuHCl)/50 mM CAPS, pH 11.0.

The Betacellulin inclusion body preparation was diluted to 60 mM GuHCl with 50 mM CAPS, pH 11.0/1 mM EDTA/1.25 mM reducing glutathione/0.5 mM oxidizing glutathione. The final protein concentration was 50–100 mg/ml by Biorad protein assay. Refolding was achieved by incubation at 4°C for 18–24h. The solution was then dialyzed against 50 mM sodium phosphate (NaP), pH 7.5, and successively filtered through 5 mm, 0.45 mm and 0.22 mm filters or subjected to 60 000 *g* centrifugation prior to cation exchange chromatography. Alternatively, the refolded material was buffer exchanged by ultrafiltration against 3 volumes of 50 mM NaP, pH 7.5.

Cleared, refolded bacterially-produced betacellulin was loaded on a cation exchange column (Bakerbond CSx) equilibrated with 40 mM NaP pH 7.0. The flow rate was 1.25 ml/min and the chromatography was carried out at room temperature. The column was washed with 20 column volumes of 40 mM NaP, pH 7.0, or until a stable baseline was achieved. The betacellulin was eluted with a 50 ml linear gradient of 0.2 –1 M NaCl in 40 mM NaP, pH 7.0. The peak fractions were at ~ 550 mM NaCl as determined by reactivity in a hemagluttinin ELISA. The peak fractions from cation exchange chromatography were pooled, diluted to 0.2 M NaCl with 40 mM NaP, pH 7.0 and applied to an FPLC TSK-heparin 5PW column (TosoHaas). The flow rate was 1 ml/min. The column was then washed with 40 mM NaP, pH 7.0 and bound protein was eluted with a 30 ml linear gradient of 0–1.0 M NaCl in 40 mM NaP, pH 7.0. The recombinant tagged betacellulin eluted at 800 mM NaCl and migrated as a single Coomassie stained band on 15% SDS-PAGE.

Betacellulin activity was measured using an EGFR tyrosine phosphorylation assay (Thorne and Plowman, 1994). Recombinant betacellulin produced as a tagged fusion construct in bacteria had an activity indistinguishable from that produced transiently in COS cells using an expression construct containing the full length human betacellulin sequence (Plowman *et al.*, in preparation).

Cell lines and cell culture

cDNAs encoding the four human erbB family receptors were subcloned into the recombinant retroviral expression vector pLXSN (Miller and Rosman, 1989), which carries a neomycin resistance gene. pLXSN and the constructs expressing the erbB family receptors were transfected into the mouse Ba/F3 pro-B-lymphocyte cell line (Palacios and Steinmetz, 1985) and selected with geneticin, generating a vector control cell line as well as clonal cell lines that express the four erbB family receptors, singly and in pairwise combinations. The generation of these cell lines has been described previously (Riese *et al.*, 1995). The cell lines used in this report are: LXSN/1 (vector control); EGFR/3; neu/12C; erbB-3/3; erbB-4/7; EGFR + neu/5D; EGFR + erbB-3/4A; EGFR + erbB-4/2A; neu + erbB-3/7A; neu + erbB-4/15A; and erbB3 + erbB-4/2B. Cells were grown in RPMI (Gibco/BRL) supplemented with 10% fetal calf serum (Sigma), 200 ug/ml G418 (Gibco/BRL), and interleukin-3 (IL-3) supplied as 10% conditioned

medium from the WEHI-3B mouse myelomonocytic leukemia cell line (Daley and Baltimore, 1988).

The ranked order of receptor expression in the double recombinant cell lines is as follows. For EGFR expression, EGFR + erbB-4/2A is higher than EGFR + neu/5D, which is higher than EGFR + erbB-3/4A. For neu expression, neu + erbB4/15A is marginally higher than EGFR + neu/5D, which is markedly higher than neu + erbB-3/7A. The levels of ErbB-3 expression are similar in the EGFR + erbB3/4A, neu + erbB-3/7A, and erbB-3 + erbB-4/2B cell lines, while the levels of erbB-4 expression are similar in the EGFR + erbB-4/2A, neu + erbB-4/15A and erbB-3 + erbB-4/2B cell lines (Riese *et al*., 1995).

Stimulation and analysis of erbB family receptor tyrosine phosphorylation

2×10^8 recombinant Ba/F3 cells were washed in phosphate-buffered saline (PBS) and resuspended in 50 ml RPMI supplemented with IL-3. The cells were incubated for 6–10 h at 37°C, washed in ice-cold PBS and resuspended in 1–3 ml ice-cold PBS. Remaining steps were performed cold or on ice. The cells were transferred in two to five 0.5 ml portions to microcentrifuge tubes. Human recombinant EGF (Collaborative Biomedical), a chemically-synthesized NRG-β 65mer (Barbacci *et al*., 1995; Riese *et al*., 1995) or recombinant human betacellulin was added at a final concentration of 100 ng/ml (EGF), 94 ng/ml (NRG-β), or 150 ng/ml (betacellulin). Following a 10 min incubation, cells were lysed and the protein content of each sample was assayed as described earlier (Riese *et al*., 1995).

Analysis of erbB family receptor tyrosine phosphorylation has been described previously (Riese *et al*., 1995). Briefly, the erbB family receptors were immunoprecipitated with antisera specific for single receptors, after which the samples were separated by electrophoresis through a 7.5% acrylamide, 0.17% bisacrylamide, 0.1% SDS gel (Sefton *et al*., 1979), electrotransferred onto nitrocellulose (DiGiovanna and Stern, 1995) and immunoblotted with the antiphosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology, Inc.). Antibody binding was detected with sheep anti-mouse coupled to horseradish peroxidase antibody NA931 (Amersham) and enhanced chemiluminescence reagents RPN2106 (Amersham).

Anti-receptor antibodies used for immunoprecipitation were as follows: antiEGFR mouse monoclonal antibody 528 (Gill *et al*., 1984); anti-Neu mouse monoclonal antibodies TAb 250 (Langton, *et al*., 1991), FSP16 (Harwerth *et al*., 1992) and TA-1 (Ab-5, Oncogene Science); anti-erbB-3 rabbit polyclonal antiserum SC-285 (Santa Cruz Biotechnology); and anti-erbB-4 rabbit polyclonal antiserum SC-283 (Santa Cruz Biotechnology). Specificity of anti-receptor antibodies has been verified previously by testing for cross-reactivity with lysates from cell lines expressing heterologous receptors (data not shown).

Stimulation and analysis of IL-3 independence

For each trial and treatment, Ba/F3 derivatives made quiescent by growth to saturation density were plated at a density of 100×10^3 cells/ml in duplicate culture dishes containing medium lacking IL-3 (IL-3-Free), medium supplemented with IL-3 (IL-3), or in medium lacking IL-3 but supplemented with 10 ng/ml human recombinant EGF (EGF–Collaborative Biomedical), 9.4 ng/ml chemically-synthesized NRG-β 65- mer (Neuregulin–(Barbacci, *et al*., 1995; Riese *et al*., 1995)), or 7 ng/ml human recombinant betacellulin (Betacellulin). Cells were stained daily with trypan blue and counted in a hemacytometer to determine viable cell densities until each sample reached a viable cell saturation density, at which time data collection was terminated. While not every cell line was tested with every factor in every trial, each combination of cell lines and factors shown was tested in a minimum of 4 trials, while some combinations were tested in as many as 20 trials. The arithmetic means and the standard error of the means of the viable cell saturation densities were calculated for each combination of cell line and treatment (Zar, 1984). Cultures exhibiting viable cell saturation densities of less than 20×10^3 cells/ml were judged to be nonresponsive to stimulation. Cultures with viable cell saturation densities of $50–400 \times 10^3$ cells/ml were judged to be exhibiting survival but not proliferation. Cultures with viable cell saturation densities of greater than 800×10^3 cells/ml were judged to be exhibiting proliferation.

Acknowledgements

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The Epidermal Growth Factor Receptor Couples Transforming Growth Factor-α, Heparin-binding Epidermal Growth Factor-like Factor, and Amphiregulin to Neu, ErbB-3, and ErbB-4*

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The epidermal growth factor (EGF) family hormones amphiregulin (AR), transforming growth factor-α (TGF-α), and heparin-binding EGF-like growth factor (HB-EGF) are thought to play significant roles in the genesis or progression of a number of human malignancies. However, the ability of these ligands to activate all four erbB family receptors has not been evaluated. Therefore, we have assessed the stimulation of erbB family receptor tyrosine phosphorylation by these hormones in a panel of mouse Ba/F3 cell lines expressing the four erbB family receptors, singly and in pairwise combinations. We also measured the stimulation of interleukin-3-independent survival or proliferation in this panel of Ba/F3 cell lines to compare the patterns of erbB family receptor coupling to physiologic responses induced by these peptides. EGF, TGF-α, AR, and HB-EGF all stimulated qualitatively similar patterns of erbB family receptor tyrosine phosphorylation and coupling to physiologic responses. Therefore, EGF, TGF-α, AR, and HB-EGF are functionally identical in this model system and behave differently from the EGF family hormones betacellulin and neuregulins.

Deregulation of the signaling network composed of the erbB family receptors and the epidermal growth factor (EGF)[1] family of peptide hormones plays an important role in a number of human metastatic diseases (reviewed in Ref. 1). Several factors contribute to the complex regulation of this system. There are four distinct erbB family receptors, including the epidermal growth factor receptor (EGFR/erbB-1), neu (erbB-2/HER2), erbB-3 (HER3), and erbB-4 (HER4). Receptor signaling can be stimulated by at least six different EGF family hormones, including EGF, transforming growth factor-α (TGF-α), amphiregulin (AR), betacellulin (BTC), heparin-binding EGF-like growth factor (HB-EGF), and the several differentially spliced variants of neuregulin (NRG), also known as neu differentiation factors, heregulins, glial growth factors, or acetylcholine receptor inducing activity (reviewed in Refs. 1–3). A single erbB family receptor (*e.g.* EGFR) can bind several different EGF family hormones (EGF, TGF-α, AR, HB-EGF) (reviewed in Refs. 1 and 2), and a single EGF family peptide (BTC) can activate more than one receptor (EGFR, erbB-4) (4). Furthermore, in cells expressing multiple erbB family receptors, EGF family peptides can induce the phosphorylation and signaling of erbB family receptors that, when expressed alone, are not phosphorylated, a process referred to as transmodulation (reviewed in Refs. 5 and 6). For example, EGF stimulates neu tyrosine phosphorylation when neu is coexpressed with the EGFR, but not when neu is expressed by itself (7–10). Indeed, with a single exception, every EGF family hormone tested thus far has the potential to regulate all four erbB family receptors through this mechanism (4, 7–19). The physiological relevance of transmodulation is supported in gene-targeting experiments in transgenic mice. Mice homozygous for disruptions in the NRG, neu, or erbB-4 genes all die *in utero* at day 10.5 and lack the trabecular extensions of the developing ventricular myocardium (20–22). The similarity of these cardiac defects suggests that NRG induction of both neu and erbB-4 signaling is required for cardiac morphogenesis. Since NRG regulates neu phosphorylation only through transmodulation (12–15, 17–19), erbB-4-dependent transmodulation of neu signaling by NRG plays a critical role in cardiac development.

Despite the relevance of erbB family receptor phosphorylation and transmodulation in regulating cellular proliferation and differentiation, the effects of TGF-α, AR, and HB-EGF on erbB family receptor signaling have not been completely evaluated. All three of these molecules stimulate EGFR phosphorylation (11, 23, 24), and both AR and TGF-α transmodulate neu (9, 11). However, the abilities of these molecules to regulate erbB-3 and erbB-4, alone and in combination with other receptors, have not been comprehensively evaluated. Consequently, while all three of these molecules stimulate EGFR signaling, they may also differentially regulate signaling by the other three erbB family receptors. This might explain quantitative differences observed in the activities of AR, HB-EGF,

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[1] The abbreviations used are: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; AR, amphiregulin; NRG, neuregulin; TGF-α, transforming growth factor-α; HB, heparin binding; BTC, betacellulin; IL, interleukin; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

and EGF (25, 26). This possibility is underscored by our recent finding that BTC not only activates EGFR, but also activates erbB-4 (4). In this report we have evaluated AR, HB-EGF, and EGF activities by stimulating IL-3-dependent Ba/F3 mouse pro-B-lymphocyte cell lines that have been engineered to express, singly and in pairwise combinations, all four erbB family receptors (4, 17).

EXPERIMENTAL PROCEDURES

Growth Factors—The production of recombinant HB-EGF has been described earlier (27). Human recombinant EGF and TGF-α were supplied by Collaborative Biomedical Products. NRG was supplied as a chemically synthesized NRG-β 65-mer (17, 28). Amphiregulin purification took advantage of the highly basic nature and high heparin affinity of AR. Due to the high isoelectric point (pI 10.1) of nonglycosylated AR, we compared AR refolding efficiency over a range of pH 7–11. Maximal activity was seen following refolding in 50 mM CAPS, pH 11.0.

AR was produced in bacteria under the control of the tightly regulated, thermoinducible bacteriophage λ PL promoter as described for betacellulin (4) with several modifications. The nucleotide sequence encoding an initiating methionine, 82 amino acids of the AR precursor (Val^{107}–Thr^{188}), and a stop codon was inserted between the unique *Bgl*II and *Xba*I sites of pPLABTC-Tag (4). The resulting plasmid was grown and transformed into competent *Escherichia coli* N4830-1. AR expression was induced and bacteria were lysed and fractionated as described previously (4). Recombinant AR was refolded in 50 mM CAPS at pH 11.0, followed by cation exchange and heparin affinity chromatography as described previously (4). AR activity was monitored using an EGFR tyrosine phosphorylation assay and an AR-specific enzyme-linked immunosorbent assay (29).

The nonglycosylated bacterial AR migrated at 14 kDa, consistent with the *N*-glycanase-treated native AR. Furthermore, it was found to inhibit the binding of ^{125}I-EGF to NRHER5 membranes (which express EGFR) as well as to live cells (29). A 50% inhibition of ^{125}I-EGF binding to NRHER5 membranes was seen at approximately 0.1 nM EGF (0.1 ng/well), 100 nM native AR or recombinant AR produced in COS cells (100 ng/well), and 150 nM bacterial AR (100 ng/well). Nonlabeled native and recombinant AR demonstrated an 85% maximal inhibition in these assays, confirming previous reports that AR has a relatively lower affinity than EGF to the EGFR.

Cell Lines and Cell Culture—The generation and characterization of the recombinant Ba/F3 cell lines used in this report have been described previously (17). These 11 cell lines, which express the four erbB family receptors singly and in pairwise combinations, are: LXSN/1 (vector control); EGFR/3; neu/12C; erbB-3/3; erbB-4/7; EGFR+neu/5D; EGFR+erbB-3/4A; EGFR+erbB-4/2A; neu+erbB-3/7A; neu+erbB-4/15A; and erbB-3+erbB-4/2B. Conditions for culturing these cell lines have been described earlier (17).

Stimulation and Analysis of ErbB Family Receptor Tyrosine Phosphorylation—The stimulation of erbB family receptor tyrosine phosphorylation in the recombinant Ba/F3 cell lines has been described previously (4, 17). Cells were stimulated with ligand at the following final concentrations: NRG, 94 ng/ml; EGF, TGF-α or HB-EGF, 100 ng/ml; AR, 1280 ng/ml. Dose-response experiments established that these hormone concentrations induced saturated levels of receptor tyrosine phosphorylation (data not shown).

Analysis of erbB family receptor tyrosine phosphorylation by immunoprecipitation and anti-phosphotyrosine immunoblotting has been described previously (4, 17). Immunoprecipitating anti-receptor antibodies were anti-EGFR mouse monoclonal antibody 528 (30), anti-Neu mouse monoclonal antibodies FSP16 (31), and TA-1 (Ab-5, Oncogene Science), anti-erbB-3 rabbit polyclonal antiserum SC-285 (Santa Cruz Biotechnology), and anti-erbB-4 rabbit polyclonal antiserum SC-283 (Santa Cruz Biotechnology). Specificity of anti-receptor antibodies has been verified by testing for cross-reactivity (data not shown) and previously we have established that hormone stimulation under these conditions does not appreciably modulate erbB family receptor protein levels (17).

RESULTS

Stimulation of Receptor Phosphorylation in Cell Lines Expressing a Single ErbB Family Receptor—In order to determine which receptors are activated individually by AR, TGF-α, and HB-EGF, we first evaluated the activation of erbB family receptor phosphorylation in the cell lines that express single erbB family receptors. Each of these factors stimulated the phosphorylation of EGFR, but not of neu or erbB-4 (Figs. 1*A* and *B*). In contrast, the positive control neuregulin-β (NRG) activated both neu and erbB-4 (Fig. 1*A*, *lane 12*; Fig. 1*B*, *lane 12*). All four EGF family peptides tested failed to stimulate erbB-3 tyrosine phosphorylation (Fig. 1*B*, *lanes 3–6*). This may be due to the minimal intrinsic kinase activity of erbB-3 (33, 34) and does not necessarily indicate that these hormones do not bind to erbB-3.

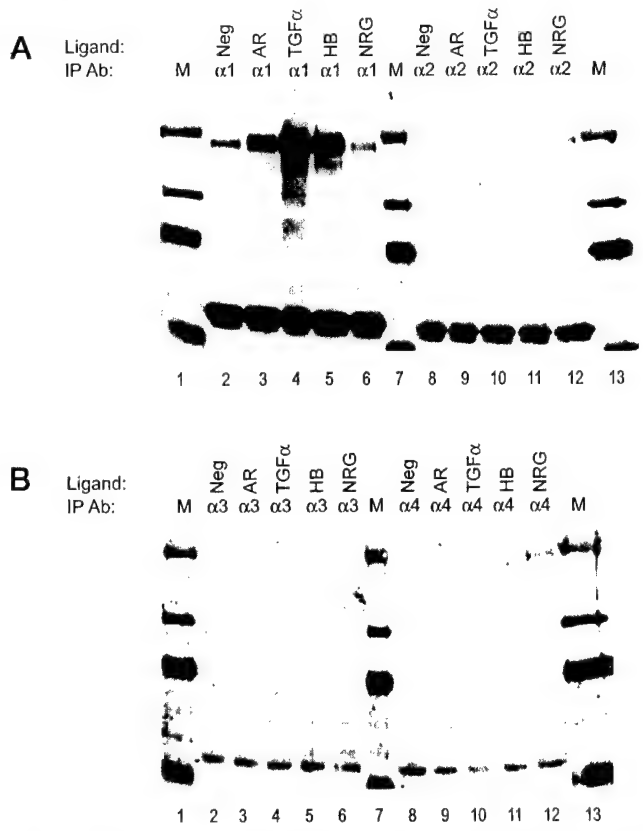

FIG. 1. **Comparison of receptor tyrosine phosphorylation in Ba/F3 derivatives expressing a single erbB family receptor and stimulated with AR, TGF-α, HB-EGF, or NRG.** Lysates from untreated or ligand-treated Ba/F3 derivatives were immunoprecipitated with anti-receptor antibodies and analyzed by immunoblotting with anti-phosphotyrosine antibody 4G10. Lysates from mock-stimulated cells are denoted *Neg*, lysates from AR-treated cells are denoted *AR*, lysates from TGF-α-treated cells are denoted *TGFα*, lysates from HB-EGF-treated cells are denoted *HB*, and lysates from NRG- treated cells are denoted *NRG*. Immunoprecipitating antibodies were *α1*, anti-EGFR; *α2*, anti-neu; *α3*, anti-erbB-3; *α4*, anti-erbB-4. *M* indicates prestained molecular mass markers (Bio-Rad), with mobilities comparable to 206, 117, 89, and 47 kDa. *A*, *lanes 2–6* are immunoprecipitates from the EGFR/3 cell line, and *lanes 8–12* are immunoprecipitates from the Neu/12C cell line. The neu tyrosine phosphorylation observed in response to NRG is apparently due to endogenous erbB-3 expression in Ba/F3 cells and is consistent with previous observations (17). *B*, *lanes 2–6* are immunoprecipitates from the ErbB-3/3 cell line, and *lanes 8–12* are immunoprecipitates from the ErbB-4/7 cell line.

In contrast, the positive control neuregulin-β (NRG) activated both neu and erbB-4 (Fig. 1*A*, *lane 12*; Fig. 1*B*, *lane 12*). All four EGF family peptides tested failed to stimulate erbB-3 tyrosine phosphorylation (Fig. 1*B*, *lanes 3–6*). This may be due to the minimal intrinsic kinase activity of erbB-3 (33, 34) and does not necessarily indicate that these hormones do not bind to erbB-3.

Stimulation of Receptor Phosphorylation in Cell Lines Expressing Two Different ErbB Family Receptors—We then evaluated erbB family receptor phosphorylation in Ba/F3 cell lines that express pairwise combinations of receptors to assess the ability of these hormones to regulate erbB-2, erbB-3, and erbB-4 through transmodulation. AR, TGF-α, and HB-EGF stimulated EGFR receptor phosphorylation in the EGFR+neu, EGFR+erbB-3, and EGFR+erbB-4 cell lines (Figs. 2*A–C*). Furthermore, these hormones induced neu and erbB-4 tyrosine phosphorylation in the EGFR+neu and EGFR+erbB-4 cell lines, respectively (Figs. 2, *A* and *C*). In contrast, TGF-α activated erbB-3 in the EGFR+erbB-3 cell line, while AR and HB-EGF did not (Fig. 2*B*).

HB-EGF, TGF-α, and AR failed to activate erbB family re-

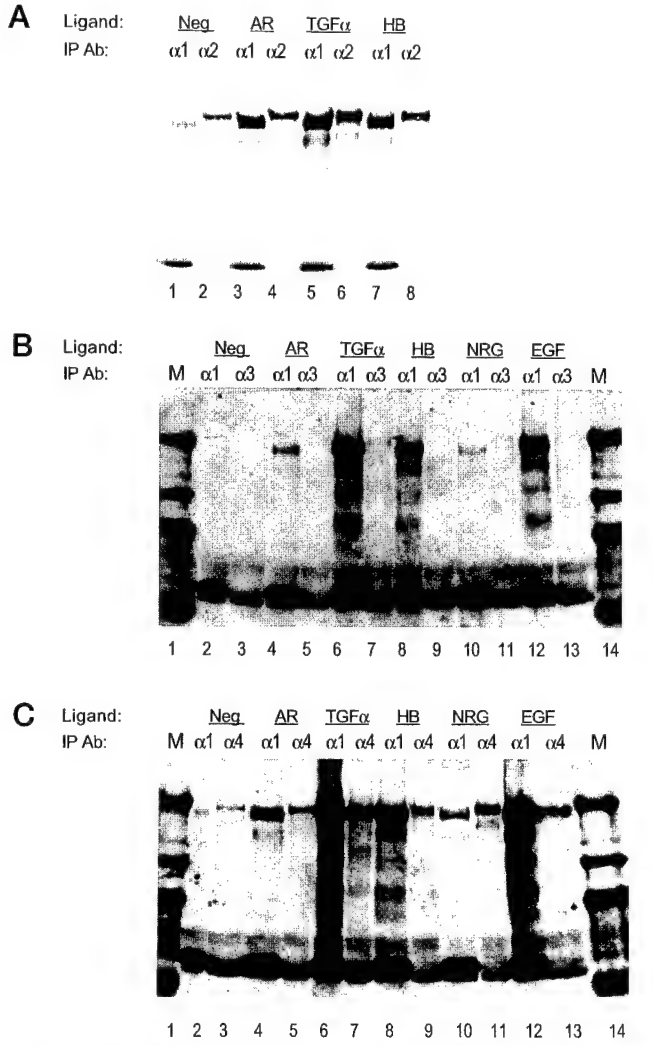

FIG. 2. **Comparison of receptor tyrosine phosphorylation in Ba/F3 derivatives expressing EGFR and another erbB family receptor and stimulated with AR, TGF-α, HB-EGF, NRG, or EGF.** Lysates from untreated or ligand-treated Ba/F3 derivatives were immunoprecipitated with anti-receptor antibodies and analyzed by immunoblotting with anti-phosphotyrosine antibody 4G10. Lysates from mock-stimulated cells are denoted *Neg*, lysates from AR-treated cells are denoted *AR*, lysates from TGF-α-treated cells are denoted *TGFα*, lysates from HB-EGF-treated cells are denoted *HB*, lysates from NRG-treated cells are denoted *NRG*, and lysates from EGF-stimulated cells are denoted *EGF*. Immunoprecipitating antibodies were *α1*, anti-EGFR; *α2*, anti-neu; *α3*, anti-erbB-3; *α4*, anti-erbB-4. *M* indicates prestained molecular mass markers (Bio-Rad), with mobilities comparable to 206, 117, 89, and 47 kDa. *A*, *lanes 1–8* are immunoprecipitates from the EGFR+neu cell line. *B*, *lanes 2–13* are immunoprecipitates from the EGFR+erbB-3 cell line. *C*, *lanes 2–13* are immunoprecipitates from the EGFR+erbB-4 cell line.

ceptors in the Neu+erbB-3, Neu+erbB-4, and ErbB-3+erbB-4 cell lines, even though NRG induced receptor phosphorylation in all three cell lines (Figs. 3*A–C*) and HB-EGF, TGF-α, and AR stimulated receptor phosphorylation in the EGFR cell line (Fig. 3*A*). Therefore, HB-EGF, TGF-α, and AR do not activate neu, erbB-3, or erbB-4 in the absence of EGFR expression and are presumably not ligands for these receptors.

Induction of IL-3-independent Survival or Proliferation in Ba/F3 Derivatives—While HB-EGF, TGF-α, and AR induced similar patterns of erbB receptor phosphorylation, another potential source of signaling specificity is that different ligands may couple the same erbB family receptors to distinct sets of cellular signaling pathways. To assess this possibility we tested whether HB-EGF, TGF-α, and AR induced different patterns of

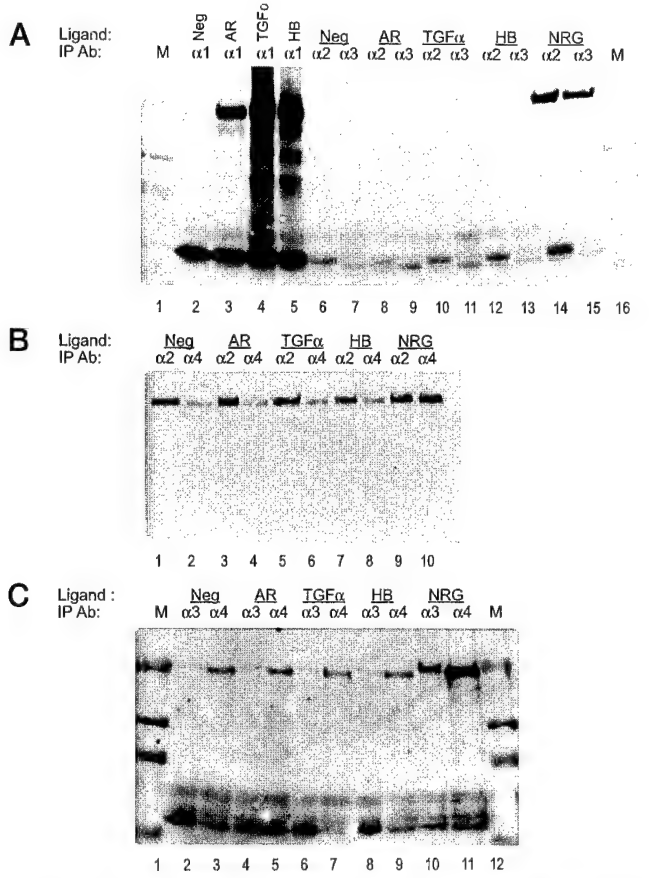

FIG. 3. **Comparison of receptor tyrosine phosphorylation in Ba/F3 derivatives expressing combinations of neu, erbB-3, or erbB-4 and stimulated with AR, TGF-α, HB-EGF, or NRG.** Lysates from untreated or ligand-treated Ba/F3 derivatives were immunoprecipitated with anti-receptor antibodies and analyzed by immunoblotting with anti-phosphotyrosine antibody 4G10. Lysates from mock-stimulated cells are denoted *Neg*, lysates from AR-treated cells are denoted *AR*, lysates from TGF-α-treated cells are denoted *TGFα*, lysates from HB-EGF-treated cells are denoted *HB*, lysates from NRG-treated cells are denoted *NRG*, and lysates from EGF-stimulated cells are denoted *EGF*. Immunoprecipitating antibodies were *α1*, anti-EGFR; *α2*, anti-neu; *α3*, anti-erbB-3; *α4*, anti-erbB-4. *M* indicates prestained molecular mass markers (Bio-Rad), with mobilities comparable to 206, 117, 89, and 47 kDa. *A*, *lanes 2–5* are immunoprecipitates from the positive control EGFR/3 cell line and *lanes 6–15* are immunoprecipitates from the Neu+erbB-3 cell line. *B*, *lanes 1–10* are immunoprecipitates from the Neu+erbB-4 cell line. The minimal stimulation of neu tyrosine phosphorylation by the positive control NRG in the neu+erbB-4 cell line is due to the high basal level of neu phosphorylation in this cell line and is consistent with previous observations (17). *C*, *lanes 2–11* are immunoprecipitates from the ErbB-3+erbB-4 cell line.

IL-3-independent survival or growth in the recombinant Ba/F3 cell lines. Our previous studies using these cell lines have established that activation of EGFR or neu, but not erbB-4, confers IL-3-independent survival, while activation of both EGFR and erbB-4 or EGFR and neu confers IL-3 independent proliferation (4, 17). As shown in Fig. 4, the vector control cell line failed to respond to any of the factors, while all four ligands induced IL-3-independent survival in the EGFR and EGFR+erbB-3 cell lines. (We judged that cultures exhibiting viable cell saturation densities of less than 10×10^3 cells/ml were nonresponsive to hormone, while cultures with viable cell saturation densities of $20–250 \times 10^3$ cells/ml were exhibiting survival but not proliferation and cultures with viable cell saturation densities of greater than 500×10^3 cells/ml were proliferating.) All four ligands stimulated IL-3-independent proliferation in the EGFR+neu and EGFR+erbB-4 cell lines.

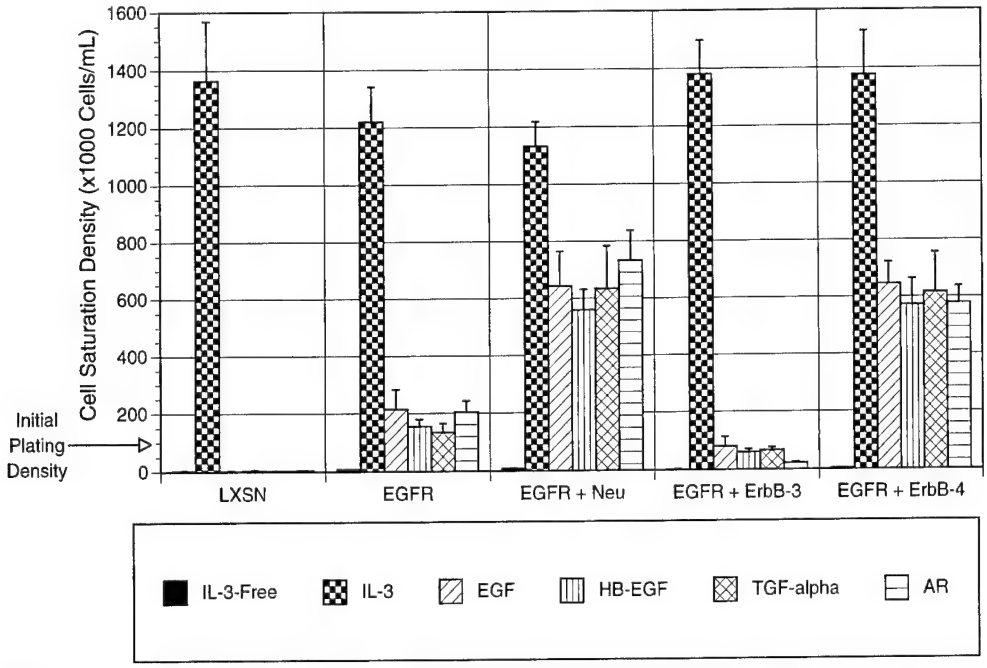

FIG. 4. **IL-3-independent saturation density of Ba/F3 cells stimulated with EGF, HB-EGF, TGF-α, or AR.** The stimulation and analysis of IL-3 independence in the recombinant Ba/F3 cell lines have been described previously (4, 17). The recombinant Ba/F3 cell lines were made quiescent by growth to saturation density and were seeded at a density of 100×10^3 cells/ml in medium lacking IL-3 (*IL-3-Free*), medium supplemented with IL-3, or in medium lacking IL-3 but supplemented with 10 ng/ml human recombinant EGF, 10 ng/ml HB-EGF, 10 ng/ml TGF-α, or 128 ng/ml AR. Cells were stained with trypan blue and counted in a hemacytometer daily 2–4 days after seeding to determine viable cell saturation densities. Each combination of cell lines and factors was tested 6–11 times and the arithmetic means of the viable cell saturation densities are indicated by the *filled bars*, while the standard errors of the means (32) are indicated by the *error bars*. Because cultures treated with IL-3-free medium exhibited viable saturation densities of less than 9×10^3 cells/ml, these values, represented by the *left-most bar* for each cell line, may not be apparent.

Consistent results were obtained in [^{3}H]thymidine incorporation assays of EGF and HB-EGF activity in the LXSN, EGFR, erbB-4, and EGFR+erbB-4 cell lines.[2] Therefore, in every cell line tested, EGF, HB-EGF, TGF-α, and AR induced growth responses that were quantitatively similar.

DISCUSSION

In this report we describe erbB family receptor tyrosine phosphorylation and coupling to cellular signaling pathways in response to the EGF family hormones AR, TGF-α, and HB-EGF. This study is the first analysis of the interactions of these hormones with the complete suite of erbB family receptors. In these assays TGF-α, AR, and HB-EGF were functionally nearly identical to each other and to EGF. These hormones stimulated only EGFR phosphorylation in the cell lines that express a single erbB family receptor, but TGF-α stimulated more EGFR phosphorylation than did HB-EGF or AR (Fig. 1). TGF-α, AR, and HB-EGF stimulated neu and erbB-4 phosphorylation in an EGFR-dependent manner (Fig. 2, *A* and *C*). However, while TGF-α stimulated erbB-3 phosphorylation in the EGFR+erbB-3 cell line, AR and HB-EGF failed to do so (Fig. 2*B*).

The reduced activity of AR relative to the other hormones has been observed in other experiments as well. EGF and TGF-α stimulate anchorage independent growth of NRK cells in the presence of transforming growth factor beta, while AR does not, and AR does not compete as well as EGF itself for ^{125}I-EGF binding to A431 cells (25). In our dose-response experiments, TGF-α stimulated markedly higher levels of EGFR phosphorylation than two independently prepared recombinant AR samples, not only in a recombinant Ba/F3 cell line, but also in ψ2 cells (an NIH/3T3-derived cell line) (35) engineered to overexpress EGFR.[3] Therefore, in our hands the reduced AR activity does not appear to be cell type- or preparation-specific, suggesting that AR may have reduced intrinsic activity compared to EGF and TGF-α. Nonetheless, AR might not be properly folded when expressed in *E. coli*, or the amino-terminal glycosylation that is present when AR is expressed in mammalian cells and is absent when AR is expressed in *E. coli* may modulate AR activity.

While we observed quantitative differences in receptor phosphorylation stimulated by TGF-α, AR, and HB-EGF, the physiologic responses to these factors were quantitatively quite similar. This was consistent with our earlier findings that the identity of receptors activated, rather than the number of activated receptors, determines the cellular response (4, 17). These factors all stimulated the IL-3-independent survival of EGFR cells and the IL-3-independent proliferation of EGFR+neu and EGFR+erbB-4 cells. In the sole exception, AR stimulated a weaker response in the EGFR+erbB-3 cell line compared to TGF-α or HB-EGF. This is concordant with the failure of AR and HB-EGF to stimulate erbB-3 phosphorylation in this cell line. These differences in TGF-α, AR, and HB-EGF activity suggests that there are threshold levels of activity required for the detection of erbB-3 phosphorylation and the efficient induction of IL-3-independent survival. Because EGFR expression in the EGFR+erbB-3 cell line is less than EGFR expression in the EGFR, EGFR+neu, and EGFR+erbB-4 cell lines (data not shown), the EGFR+erbB-3 cell line is likely to be more sensitive to the reduced activity of AR and HB-EGF.

The specification of biological responses to the EGF family/erbB receptor family signaling network is regulated at several distinct levels. We have previously demonstrated that the EGF

[2] K. Elenius and M. Klagsbrun, unpublished results.

[3] D. J. Riese II and D. F. Stern, unpublished results.

TABLE I

Regulation of erbB family receptor signaling and coupling to cellular responses by EGF family ligands

Results are abstracted from Riese *et al.* (4, 17) and Figs. 1–4 of this report.

Cell Line	Receptor	Group I EGF, AR, TGFα, HB-EGF		Group II NRGs		Group III BTC	
		Tyr phos[a]	Growth[b]	Tyr phos[a]	Growth[b]	Tyr phos[a]	Growth[b]
EGFR		+	S	−	N	+	S
Neu		−	N	+[c]	S[c]	−	N
ErbB-3		−	NT[d]	−	N	−	N
ErbB-4		−	NT[d]	+	N	+	N
EGFR+Neu	EGFR	+	P	*[c]	S[c]	+	P
	Neu	+		*[c]		+	
EGFR+erbB-3	EGFR	+	S	+	N	+	S
	ErbB-3	+		+		+	
EGFR+erbB-4	EGFR	+	P	+	P	+	P
	erbB-4	+		+		+	
Neu+erbB-3	Neu	−	NT[d]	+	S	−	N
	ErbB-3	−		+		−	
Neu+erbB-4	Neu	−	N[e]	+	S	+	S
	ErbB-4	−		+		+	
erbB-3+erbB-4	ErbB-3	−	N[e]	+	N	−	N
	ErbB-4	−		+		+	

[a] No increase in receptor tyrosine phosphorylation (Tyr phos) is indicated by "−," increased receptor tyrosine phosphorylation is indicated by "+," and ambiguity due to high basal levels of receptor phosphorylation is indicated by "*."

[b] The absence of an IL-3-independent response is indicated by "N," stimulation of IL-3 independent survival is indicated by "S," and stimulation of IL-3 independent proliferation is indicated by "P." "NT" indicates not tested.

[c] The responses to NRGs are apparently due to interactions with the endogenous erbB-3 in Ba/F3 cells (17).

[d] Given the absence of receptor tyrosine phosphorylation, no IL-3 independent response is expected.

[e] Results for EGF only. AR, TGF-α, and HB-EGF were not tested, but a negative response to these ligands identical to that observed for EGF is anticipated because none of these hormones activated receptor phosphorylation in these cell lines.

family of hormones can be divided into three groups according to their ability to stimulate phosphorylation of different erbB family receptors. The data presented here permit us to assign TGF-α, AR, and HB-EGF into these three functional groups (Table I). Group I of EGF family hormones now consists of EGF, TGF-α, AR, and HB-EGF. These peptides activate the EGFR in the absence of additional erbB family receptor expression and can stimulate the tyrosine phosphorylation of neu, erbB-4, and in some cases, erbB-3, in an EGFR-dependent manner. Group II of EGF family hormones consists of the NRGs. These peptides activate erbB-4 in the absence of additional erbB family receptor expression. However, the NRGs also bind to erbB-3, and activate it when coexpressed with any other erbB family receptor, and can stimulate neu or EGFR phosphorylation when these receptors are coexpressed with either erbB-3 or erbB-4. Group III of EGF family hormones consists solely of BTC. BTC activates EGFR or erbB-4 in the absence of additional erbB family receptor expression. Moreover, BTC stimulates neu tyrosine phosphorylation when neu is coexpressed with EGFR or erbB-4, and activates erbB-3 when erbB-3 is coexpressed with EGFR, but not when erbB-3 is coexpressed with erbB-4. Consequently, with one exception, hormonal activation of a single erbB family receptor can transmodulate any of the other three erbB family receptors. In different cells distinct EGF family hormones activate discrete sets of erbB family receptors, and biological responses to these hormones are specified in part by which erbB family receptors are expressed.

A corollary is that the activation of different erbB family receptors results in distinct biological responses (34, 36–46). We have observed such distinctions in our system. While EGFR activation results in the IL-3-independent survival of Ba/F3 cells and erbB-4 activation does not result in an IL-3-independent response, activation of both EGFR and erbB-4 results in IL-3-independent proliferation. Similarly, while activation of EGFR or neu alone results in IL-3-independent survival, activation of both EGFR and neu results in IL-3-independent proliferation (Table I). EGFR+erbB-4 cells stimulated with EGF, NRG, or BTC all undergo IL-3-independent proliferation, suggesting that the mechanism by which the receptors are activated does not appear to specify distinct biological responses. The stimulation of distinct biological responses by the four erbB family receptors apparently occurs through coupling of different sets of downstream signaling proteins to each receptor. Distinct sets of tyrosine phosphorylated proteins are coimmunoprecipitated with activated EGFR or activated erbB-4 (4). Experiments to identify these phosphoproteins and to determine their functional role in mediating erbB family receptor signaling are underway.

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Cripto Enhances the Tyrosine Phosphorylation of Shc and Activates Mitogen-activated Protein Kinase (MAPK) in Mammary Epithelial Cells*

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Cripto-1 (CR-1), a recently discovered protein of the epidermal growth factor (EGF) family, was found to interact with a high affinity, saturable binding site(s) on HC-11 mouse mammary epithelial cells and on several different human breast cancer cell lines. This receptor exhibits specificity for CR-1, since other EGF-related peptides including EGF, transforming growth factor α, heparin-binding EGF-like growth factor, amphiregulin, epiregulin, betacellulin, or heregulin β1 that bind to either the EGF receptor or to other type 1 receptor tyrosine kinases such as *erb* B-3 or *erb* B-4 fail to compete for binding. Conversely, CR-1 was found not to directly bind to or to activate the tyrosine kinases associated with the EGFR, *erb* B-2, *erb* B-3, or *erb* B-4 either alone or in various pairwise combinations which have been ectopically expressed in Ba/F3 mouse pro-B lymphocyte cells. However, exogenous CR-1 could induce an increase in the tyrosine phosphorylation of 185- and 120-kDa proteins and a rapid (within 3–5 min) increase in the tyrosine phosphorylation of the SH2-containing adaptor proteins p66, p52, and p46 Shc in mouse mammary HC-11 epithelial cells and in human MDA-MB-453 and SKBr-3 breast cancer cells. CR-1 was also found to promote an increase in the association of the adaptor Grb2-guanine nucleotide exchange factor-mouse *son of sevenless* (mSOS) signaling complex with tyrosine-phosphorylated Shc in HC-11 cells. Finally, CR-1 was able to increase $p42^{erk-2}$ mitogen-activated protein kinase (MAPK) activity in HC-11 cells within 5–10 min of treatment. These data demonstrate that CR-1 can function through a receptor which activates intracellular components in the *ras/raf*/MEK/MAPK pathway.

The human cripto-1 (CR-1)[1] gene (also known as teratocarcinoma-derived growth factor-1 (TDGF-1)) is located on chromosome 3p21·3 and codes for a 28–36-kDa glycoprotein of 188 amino acids. CR-1 possesses an epidermal growth factor (EGF)-like consensus sequence that contains six cysteine residues in a region of approximately 37 amino acids (1). However, unlike other peptides within this family of growth factors that have a three-looped EGF-motif (designated A, B, and C) which are formed by three intramolecular disulfide bonds, the EGF-like repeat in CR-1 lacks an A loop and the B loop is truncated (2, 3). In addition, unlike most growth factors in the EGF family, the human CR-1 protein lacks a conventional hydrophobic signal peptide and a classical hydrophobic transmembrane domain (2). Nevertheless, recombinant human CR-1 protein is secreted when transiently expressed in Chinese hamster ovary cells (3). Refolded CR-1 peptides that correspond to the EGF-like repeat within the human CR-1 protein are mitogenic for nontransformed and malignant human and mouse mammary epithelial cells (3). Human CR-1 can also function as a dominant transforming gene *in vitro* in mouse NIH-3T3 fibroblasts and in mouse NOG-8 mammary epithelial cells (1, 4). CR-1 mRNA and immunoreactive CR-1 protein are differentially expressed in several human breast cancer cell lines, in approximately 80% of primary human infiltrating breast tumors and in 50% of ductal carcinomas *in situ* (5, 6).

Peptide growth factors that are members of the EGF family include transforming growth factor α (TGFα), amphiregulin (AR), heparin-binding EGF-like growth factor, betacellulin (BTC), epiregulin, and the neuregulin subfamily that consists

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[1] The abbreviations used are: CR-1, cripto-1; EGF, epidermal growth factor; TGFα, transforming growth factor α; AR, amphiregulin; HRG, heregulin; BTC, betacellulin; EGFR, EGF receptor; mSOS, mouse *Son of Sevenless*, gunanine nucleotide exchange factor; MAPK, mitogen-activated protein kinase; *erk*, extracellular signal-regulated protein kinase; MEK, mitogen-activated *erk*-activating kinase; MBP, myelin basic protein; Shc, *Src* homologous and collagen protein; Grb2, growth factor receptor-bound protein; PAGE, polyacrylamide gel electrophoresis; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

of various isoforms of α and β heregulin (HRG), gilial cell growth factors, and acetycholine receptor inducing activity (7–10). The different neuregulins are derived by alternative splicing from a single gene. Peptides in the EGF family bind to and activate members of the *erb* B family of type 1 receptor tyrosine kinases. EGF, TGFα, AR, heparin-binding EGF-like growth factor, and epiregulin bind exclusively to the epidermal growth factor receptor (*erb* B/EGFR), whereas the neuregulin subfamily of peptides bind to c-*erb* B-3/HER-3 or c-*erb* B-4/HER-4 that can then heterodimerize and activate c-*erb* B-2/HER-2 following transphosphorylation (11–15). BTC can bind to either the EGFR or c-*erb* B-4. Since ligand-dependent activation of the EGF receptor can also lead to heterodimerization with c-*erb* B-2, c-*erb* B-3, or c-*erb* B-4, this suggests that different pairs of heterodimers within the type 1 receptor tyrosine kinase family may contribute to the array of responses to various EGF-like ligands in a cell-specific and possibly ligand-specific manner by the recruitment of different combinations of intracellular signaling proteins (14). This apparent redundancy of different ligands and receptors may therefore contribute to signal diversification and amplification. One major pathway that is activated by a several different EGF-like ligands through these type 1 receptor tyrosine kinases is the *ras/raf*/mitogen-activated protein kinase (MAPK) signal transduction pathway (16).

The HC-11 mouse mammary epithelial cell line is a clonal isolate originally derived from the COMMA-1D cell line, which was established from a midpregnant Balb/c mouse mammary gland (17). These cells express a number of mammary epithelial markers such as β-casein and express several distinct type 1 receptor tyrosine kinases, including the EGFR, c-*erb* B-2, and c-*erb* B-3 (17–20). In this context, HC-11 cells have been useful as an *in vitro* model system in which to define the intracellular signaling pathways that are engaged by EGF-related peptides that utilize these type 1 receptor tyrosine kinases and are involved in regulating proliferation and differentiation in mammary epithelial cells (19, 20). In the present study, we have attempted to define the intracellular signaling pathway that might be activated in HC-11 cells and in several different human breast cancer cell lines by a 47-mer refolded CR-1 peptide that corresponds in sequence to the EGF-like repeat of the human CR-1 protein (3). The CR-1 related peptide was found to bind to a unique specific, high-affinity receptor that is not the EGFR, *erb* B-2, *erb* B-3, or *erb* B-4. CR-1 was also found to enhance the tyrosine phosphorylation of the SH2-adaptor protein, Shc, and to promote the association of Grb2-mSOS intracellular signaling complex with tyrosine-phosphorylated Shc. These events were subsequently related to the downstream activation of the $p42^{erk\text{-}2}$ MAPK isoform.

MATERIALS AND METHODS

Cell Culture and Growth—HC-11 mouse mammary epithelial cells were routinely grown in RPMI 1640 medium containing 8% heat-inactivated fetal bovine serum, 5 μg/ml bovine insulin (Sigma), and 10 ng/ml EGF (Collaborative Research, Watham, MA). Human breast cancer cell lines, MCF-7, ZR-75-1, T-47D, MDA-MB-231, MDA-MB-453, and SKBr-3 were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and insulin (10 μg/ml) as described previously (21). The Ba/F3 mouse pro-B lymphocyte cell line and clonal derivatives that express individual or pairs of type 1 receptor tyrosine kinases were generated as described previously (22, 23). Cells were treated with different concentrations of either refolded p47 CR-1 peptide that corresponds in sequence to the EGF-like motif in human CR-1 (3), recombinant human TGFα (Bachem), recombinant human AR, recombinant human HB-EGF, recombinant human BTC (R & D Systems, Inc.), recombinant mouse epiregulin (generously supplied by Toshi Komuraski, Taisho Pharmaceutical Co., Saitama, Japan), or recombinant human HRG $\beta1_{177\text{-}244}$ (generously supplied by Mark Sliwkowski, Genentech, Inc., South San Francisco, CA).

Immunoprecipitation and Western Blot Analysis—Cells were grown until they were subconfluent and then propagated in serum-free medium containing human transferrin (10 μg/ml) and type 1V Pedersen fetuin (1 mg/ml) for 48 h. Cells were treated in serum-free medium with different concentrations of p47 CR-1 for various times and then lysed in 20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 5 mM $MgCl_2$, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 20 mM sodium fluoride. In some cases the clarified protein lysates were either immunoprecipitated (0.5 mg/sample) with 2 μg of a rabbit anti-Shc antibody (Transduction Laboratories, Lexington, KY) or with 2 μg of anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology, Inc.). Crude protein lysates (25 μg/sample) or immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, blocked with 2% dry milk in Tris-buffered saline with 0.05% Tween 20, and incubated with a 1:1000 dilution of anti-Shc, anti-Grb-2, or anti-mSOS monoclonal antibodies (Transduction Laboratories) or a 1:2000 dilution of the anti-phosphotyrosine monoclonal antibodies 4G10 and/or PY-20 (ICN Pharmaceuticals, Inc., Costa Mesa, CA). The bound mouse monoclonal antibodies were detected using a 1:5000 dilution of goat anti-mouse IgG conjugated to horseradish peroxidase (Amersham). Immunoreactive bands were detected by enhanced chemiluminescence (ECL, Amersham). *erb* B receptors were immunoprecipitated from recombinant Ba/F3 clonal derivatives using monospecific antisera as described previously (22).

MAPK Assays—A rabbit anti-MAPK antibody (Upstate Biotechnology, Inc., Lake Placid, NY) at a 1:1000 dilution that recognizes both extracellular signal-regulated protein kinase *erk-1* and *erk-2* MAPK was used to detect activation of immunoreactive MAPK proteins by means of band shift following SDS-PAGE of crude cellular lysates as described previously (24). Bands were detected by the colorimetric NBT/BCIP system (Kirkegaard Perry Labs, Gaithersburg, MD). Alternatively, cells were lysed in buffer containing 1% Nonidet P-40, 0.5% deoxycholate, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM sodium orthovanadate, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Lysates (100 μg/sample) were immunoprecipitated with an anti-*erk-1* rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After 2 h, the immunoprecipitates were washed twice with lysis buffer followed by a final wash in kinase buffer containing 30 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM dithiothreitol. After the final wash, the immunoprecipitates were suspended in 30 μl of kinase buffer containing 5 μg of myelin basic protein (MBP) and 5 μCi of [γ-^{32}P]ATP (1000 Ci/mmol, Amersham) and incubated for 30 min at 37 °C. The reaction was stopped with 2 × SDS sample buffer. The samples were then run on a 10% Tricine gel followed by autoradiography. The MBP bands were quantified with a densitometer (Molecular Dynamics) (19).

Ligand Binding Assays—^{125}I-p47 CR-1 peptide was prepared using IODO-GEN (Pierce Chemical Co.) in which 5 μg of peptide was labeled with 1 mCi of $Na^{125}I$ to a specific activity of 5–10 μCi/μg. Monolayers of HC-11 cells or human breast cancer cells in 12-well cluster dishes were washed in serum-free RPMI 1640 or Dulbecco's modified Eagle's medium containing 20 mM HEPES (pH 7.4) and 0.1% bovine serum albumin and treated in the same medium. Cells were incubated at 23 °C for 2 h in 1 ml of binding buffer containing 3 × 10^5 cpm of ^{125}I-p47 CR-1 in the absence or presence of increasing concentrations of unlabeled p47 CR-1 or with other EGF-related peptides. Cells were washed twice with phosphate-buffered saline and then solubilized in 1 ml of buffer containing 10 mM Tris-HCl (pH 8.0) and 0.5% SDS and counted in a γ-counter. The binding data were analyzed using the Ligand program to determine the K_d value as described (25).

RESULTS

Binding of CR-1 to HC-11 Mouse Mammary Epithelial Cells and Human Breast Cancer Cells—A 47-mer CR-1 refolded peptide that contains the EGF-like motif of human CR-1 is able to stimulate the proliferation and to inhibit β-casein expression in mouse HC-11 mammary epithelial cells.[2] To determine if a high-affinity, specific binding site is expressed on HC-11 mammary epithelial cells that might mediate the biological effects of CR-1, HC-11 cells were incubated with ^{125}I-p47 CR-1 in the absence or presence of increasing concentrations of unlabeled p47 CR-1. As illustrated in Fig. 1*A*, there is specific binding of the ^{125}I-p47 CR-1 peptide to HC-11 cells with a K_d of approxi-

[2] S. Kannan, manuscript in preparation.

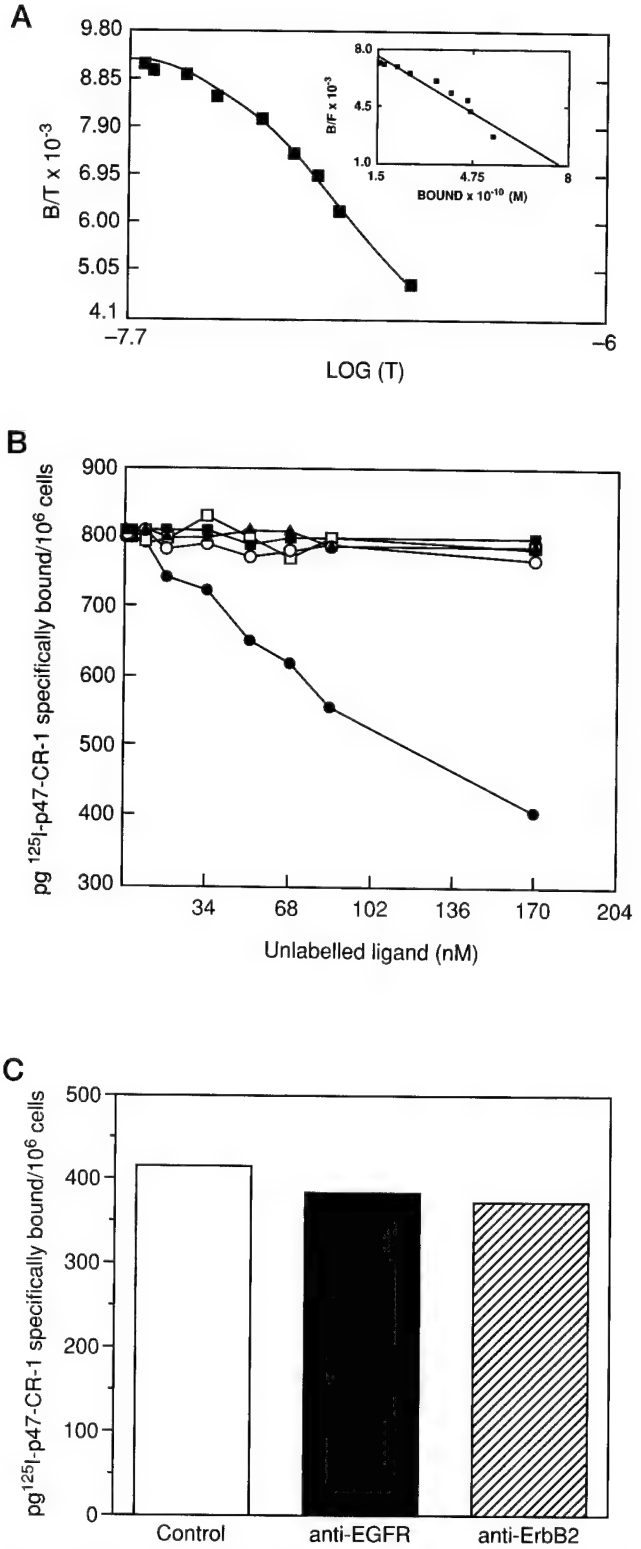

FIG. 1. **Binding and competition curve of ^{125}I-p47 CR-1.** Binding of ^{125}I-p47 CR-1 to HC-11 cells (*A*); *insert*, Scatchard plot of binding isotherm. *B*, competition of specific binding of ^{125}I-p47 CR-1 to HC-11 cells by various concentrations of EGF (○), AR (□), TGFα (■), HRGβ1 (▲), or p47 CR-1 (●). *C*, MDA-MB-231 human breast cancer cells were treated with 25 μg/ml anti-EGFR monoclonal antibody or 25 μg/ml anti-*erb* B-2 TAb 250 monoclonal antibody and specific binding of ^{125}I-p47 CR-1 (10 ng/ml) was determined.

mately 96 nM (Fig. 1*A*, *inset*). Various concentrations of EGF, TGFα, AR, or HRGβ1 were unable to compete for binding with ^{125}I-p47 CR-1 on HC-11 cells (Fig. 1*B*). Likewise, heparin-binding EGF-like growth factor, epiregulin, or BTC were ineffective in blocking the binding of the ^{125}I-p47 CR-1 peptide (data not shown). This potential receptor is not unique to HC-11 mouse mammary epithelial cells since ^{125}I-p47 CR-1 also binds specifically and with high affinity to several estrogen receptor positive (MCF-7, T47-D, and ZR-75–1) and to estrogen receptor negative (MDA-MB-231, MDA-MB-453 and SKBr-3) human breast cancer cell lines that exhibits comparable specificity for CR-1 binding as in mouse HC-11 cells (Table I). Moreover, blocking the EGFR receptor on MDA-MB-231 human breast cancer cells with 25 μg/ml of the 528 anti-EGFR monoclonal blocking antibody which is sufficient to impede ^{125}I-EGF binding (26) does not lead to any change in the specific binding of ^{125}I-p47 CR-1 to these cells (Fig. 1*C*), confirming the observation that CR-1 does not directly bind to the EGFR (3). In addition, the binding of ^{125}I-p47 CR-1 peptide to MDA-MB-231 cells or SKBr-3 cells (data not shown) is unaffected by the anti-*erb* B-2 TAb 250 blocking monoclonal antibody.

TABLE I
Characteristics of CR-1 binding to mouse and human mammary epithelial cells

Data were obtained from appropriate Scatchard plots using various concentrations of unlabeled p47 CR-1 with ^{125}I-p47 CR-1.

Cell line	nM	Sites/cell
HC-11	96	3.9×10^5
MDA-MB-453	80	4.4×10^5
MDA-MB-231	33	1.8×10^5
SKBr 3	78	4.5×10^5
ZR-75–1	61	1.1×10^5
T47-D	50	1.4×10^5
MCF-7	26	5.3×10^4

CR-1 Fails to Directly Activate erb B Family Receptor Tyrosine Phosphorylation in Recombinant Ba/F3 Cells—Parental Ba/F3 mouse pro-B lymphocytes express low levels of endogenous *erb* B-3 but do not express EGFR, *erb* B-2, or *erb* B-4. Ba/F3 clones have been genetically engineered to ectopically express either EGFR, *erb* B-2, *erb* B-3, or *erb* B-4 type 1 receptor tyrosine kinases either alone or in different pairwise combinations (22, 23). These recombinant cell lines have been particularly useful in defining different homodimeric and heterodimeric combinations of the type 1 receptor kinases that can bind to various ligands within the EGF family of peptides (23). Since CR-1 is structurally related in part to this family of peptides, the 47-mer CR-1 refolded peptide and an Sf9-derived recombinant human GST-CR-1 fusion protein[3] were tested for their ability to directly bind and stimulate receptor tyrosine phosphorylation in single recombinant Ba/F3 derivatives that are expressing different *erb* B family members (Fig. 2). p47 CR-1 was unable to significantly modify EGFR (Fig. 2*A*), *erb* B-2/*neu*/HER-2 (Fig. 2*B*), *erb* B-3/HER-3 (Fig. 2*D*), or *erb* B-4/HER-4 (Fig. 2*C*) tyrosine phosphorylation in Ba/F3 clones that were expressing these tyrosine kinases. In contrast, BTC was able to stimulate EGFR and *erb* B-4 phosphorylation (Fig. 2, *A* and *C*) while HRGβ1 was able to indirectly stimulate *erb* B-2 phosphorylation (Fig. 2*B*) by binding to endogenous *erb* B-3 (22). p47 CR-1 was also ineffective in modulating the tyrosine phosphorylation of these different type 1 receptor tyrosine kinases in Ba/F3 cells that were expressing different pairs of *erb* B receptors demonstrating that CR-1 does not directly bind to heterodimers within this family of receptor tyrosine kinases (data not shown). Similar negative results on tyrosine receptor phosphorylation in either single or double *erb* B expressing recombinant Ba/F3 clones were obtained after treatment of

[3] M. Seno, manuscript in preparation.

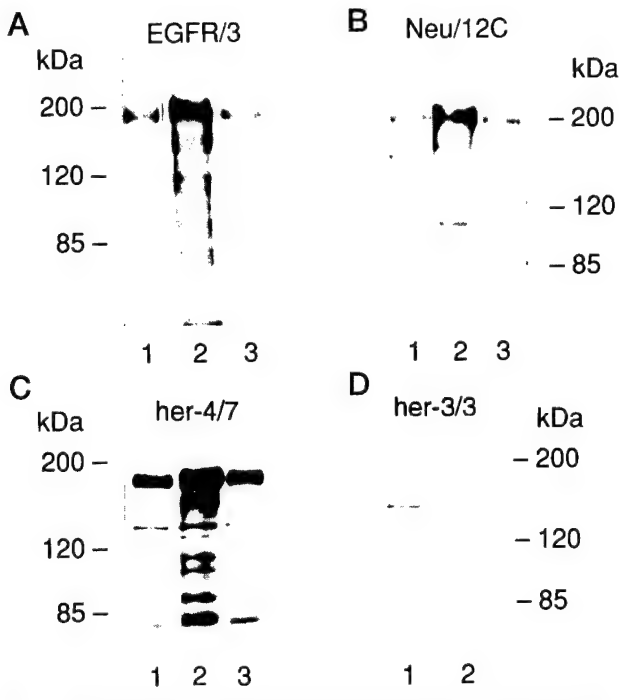

FIG. 2. **Modulation of *erb* B receptor tyrosine phosphorylation by CR-1 in recombinant Ba/F3 clonal derivatives.** Ba/F3 clones that were expressing individual *erb* B family members including the EGFR (*A*), *erb* B-2(*B*), *erb* B-4 (*C*), or *erb* B-3 (*D*) were treated without (*lane 1* in *A–D*) or with 100 ng/ml p47 CR-1 (*lane 3* in *A–C* or *lane 2* in *D*), BTC (*lane 2* in *A* and *C*), or HRGβ1 (*lane 2* in *B*) for 10 min. Cell lysates were then immunoprecipitated with monospecific anti-receptor antibodies and analyzed by immunoblotting with anti-phosphotyrosine antibodies.

these cells with the full-length Sf9 recombinant GST-human CR-1 fusion protein.

CR-1 Stimulates Tyrosine Phosphorylation of Shc and Activates MAPK in HC-11 Mammary Epithelial Cells and in Human Breast Cancer Cells—Although CR-1 does not directly activate any known type 1 receptor *erb* B tyrosine kinase, its receptor may still consist of a membrane-associated tyrosine kinase or alternatively the receptor may associate with a another cytoplasmic or membrane bound tyrosine kinase to generate a signal. To determine if the p47 CR-1 peptide could modulate the tyrosine phosphorylation of other proteins in HC-11 cells, serum-starved cells were treated with p47 CR-1 for 5 min and cell lysates were immunoprecipitated with 4G10 mouse monoclonal anti-phosphotyrosine antibody. Immunoprecipitates were then electrophoresed and probed by Western blot analysis with the PY-20 mouse monoclonal anti-phosphotyrosine antibody (Fig. 3*A*). An increase in tyrosine phosphorylation of a 185-kDa protein was observed. A similar CR-1-induced increase in the tyrosine phosphorylation of a 185-kDa protein was also observed in other cells that were capable of binding ^{125}I-p47 CR-1 such as MDA-MB-453, SKBr3, and T47-D human breast cancer cells after immunoprecipitation and Western blotting with anti-phosphotyrosine antibodies (data not shown). The identity of the p185 phosphoprotein has not yet been identified.

Shc is an SH-2 containing adaptor protein that exists as three distinct isoforms of 66, 52, and 46 kDa and that becomes tyrosine-phosphorylated after ligand activation of several different type 1 receptor tyrosine kinases (27–29). Cell lysates from p47 CR-1-treated serum-starved HC-11 cells exhibited a time-dependent increase in the tyrosine phosphorylation of the p66 Shc and p46 Shc isoforms, which could be detected following immunoprecipitation with an anti-Shc antibody and subsequent screening in Western blot analysis with anti-phosphotyrosine antibodies (Fig. 3, *B* and*C*). In HC-11 cells which had been sufficiently serum-starved, the increase in phosphorylation of p46 Shc (Fig. 3*B*) and p66 Shc (Fig. 3*C*) in response to p47 CR-1 was transient with an 8-fold increase in p46 Shc phosphorylation being observed after 5–7 min, which decreased to control levels after 10 min of treatment (Figs. 3*B* and 5*B*). No significant changes in the phosphorylation of the p52 isoform of Shc were detected in HC-11 cells. A less dramatic but measurable increase in the tyrosine phosphorylation of either p52 and/or p46 Shc could also be detected in either MDA-MB-453 or SKBr-3 human breast cancer cells after treatment with either the p47 CR-1 peptide (Fig. 3*C*) or the GST-CR-1 recombinant fusion protein (data not shown). Proteins of 185 and 120 kDa that were also tyrosine-phosphorylated in a transient manner were detected in the Shc immunoprecipates after probing the Western blots with a mixture of two different anti-phosphotyrosine monoclonal antibodies (Fig. 3*C*). These proteins were found to co-immunoprecipitate with Shc after stimulation of HC-11, MDA-MB-453, or SKBr-3 cells with p47 CR-1. The identity of these two proteins has not been determined.

Shc is an adaptor protein that can bind to specific consensus sequences which contain phosphotyrosine residues in the COOH terminus of several different tyrosine kinase receptors through either an SH2 domain or a phosphotyrosine-binding domain (29). In turn, binding of phosphorylated Shc to the SH2 domain of Grb2 through the tyrosine-phosphorylated collagen homology domain of Shc can link a number of different growth factor receptor tyrosine kinases to the *ras/raf*/MEK/MAPK signaling pathway since Grb2 is intrinsically complexed with the *ras* guanine nucleotide exchanger, SOS (27, 29). To ascertain if p47 CR-1 could facilitate the association of the Grb2-mSOS complex with phosphorylated Shc in mammary epithelial cells, HC-11 cell lystates were immunoprecipated with the Shc antibody and immunoblotted with either an anti-Grb2 (Fig. 4*A*) or an anti-SOS (Fig. 4*B*) antibody. In both cases, p47 CR-1 induced a time-dependent increase in the association of Grb2 and mSOS with phosphorylated Shc. Activation of p21^{ras} by SOS can ultimately lead to the downstream activation of mitogen-activated, *erk*-activating kinase (MEK) through *raf* and the subsequent stimulation of MAPK activity (16, 30). To ascertain if p47 CR-1 could stimulate MAPK activity, serum-starved HC-11 cells were treated for different times with the peptide, and cell lysates were electrophoresed and probed with an anti-MAPK antibody that detects both MAPK isoforms, p44$^{erk\text{-}1}$ and p42$^{erk\text{-}2}$ (Fig. 5*A*). Alternatively, cell lysates were immunoprecipitated with an anti-MAPK antibody and utilized in an *in vitro* kinase assay with MBP as a substrate to quantify total MAPK activity (Fig. 5*B*). The p47 CR-1 peptide produced a rapid tyrosine phosphorylation of p42$^{erk\text{-}2}$ within 3–5 min that could be detected by the presence of a slower migrating band that represents the phosphorylated form of p42$^{erk\text{-}2}$ (Fig. 5*A*). Phosphorylation of this species of p42$^{erk\text{-}2}$ peaked at 7 min and subsequently declined. A nearly 4-fold increase in phosphorylation of the MAPK substrate, MBP, was also observed and found to have identical kinetics in response to p47 CR-1 treatment and was delayed relative to the increase in p46 Shc phosphorylation (Fig. 5*B*).

DISCUSSION

EGF, TGFα, AR, and HRGβ1 stimulate the growth and regulate the differentiation of normal and malignant mouse and human mammary epithelial cells *in vitro* (7, 11, 31–34). However, the role of other EGF-related peptides in regulating the growth and differentiation of mammary epithelial cells has not been fully explored. This may be particularly important since only a subset of peptides within the EGF family bind exclu-

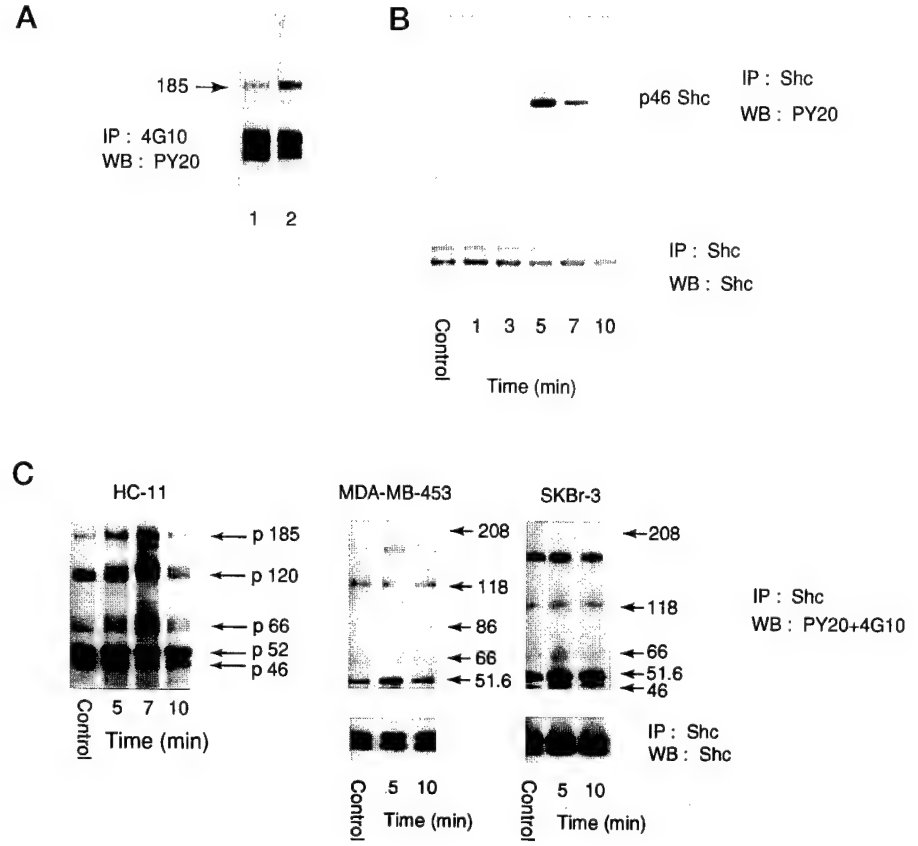

FIG. 3. **Tyrosine phosphorylation of Shc after CR-1 treatment.** Serum-starved HC-11 (*A-C*) or MDA-MB-453 or SKBr-3 human breast cancer cells (*C*) were treated without or with 100 ng/ml p47 CR-1 for 5 min. *A, lane 2*, or various times indicated (*B* and *C*). The cell lysates were immunoprecipitated with anti-phosphotyrosine (4G10, Upstate Biotechnologies) antibody (*A*) or a polyclonal anti-Shc antibody (*B* and *C*). The immunoprecipitates (*IP*) were resolved on a 8–16% SDS-PAGE gel and immunoblotted with a PY20 antibody (*A* and *B, upper panel*) or a mixture of PY20 and 4G10 antibodies (*C*). The *lower panels* in *B* and *C* represent the same blots stripped and reprobed with monoclonal anti-Shc antibody to demonstrate that equal amounts of Shc are present in all lanes.

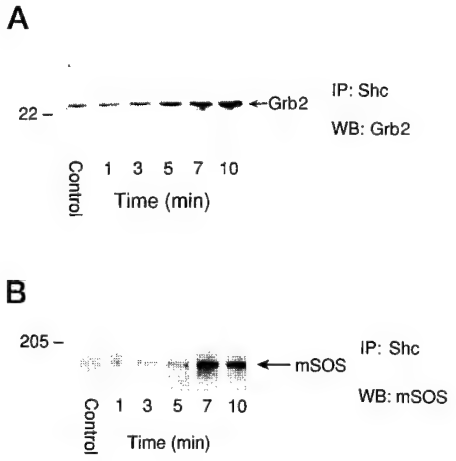

FIG. 4. **Enhanced Grb2-mSOS association with Shc upon CR-1 stimulation in HC-11 cells.** HC-11 cells were treated without or with p47 CR-1 (100 ng/ml) for various times. The cell lysates were immunoprecipated (*IP*) with polyclonal anti-Shc antibody and analyzed by Western blotting using an anti-Grb2 (*A*) or anti-mSOS (*B*) antibody.

sively to the EGFR. Additional proteins in this family, such as the neuregulin subfamily which include the HRGs, bind to other members of the type 1 receptor tyrosine kinase family of receptors such as c-*erb* B-3 or c-*erb* B-4, that can then heterodimerize and activate c-*erb* B-2 following transphosphorylation (3–15, 31, 34–36). Finally, BTC can equally activate either the EGFR or c-*erb* B-4 (23). Since ligand-dependent activation of the EGF receptor can also lead to heterodimerization with c-*erb* B-2, c-*erb* B-3, or c-*erb* B-4, this suggests that different combinatorial pairs of heterodimers within the type 1 receptor tyrosine kinase family may contribute in a hierarchical fashion through signial diversification to the array of responses that are produced to various EGF-like ligands in a cell-specific manner (14, 15, 35, 36).

The present study is the first to demonstrate that a refolded peptide which corresponds in sequence to the EGF-like motif of the human CR-1 protein can bind to a unique receptor that can modify the tyrosine phosphorylation of different proteins which are components in the *ras/raf*/MEK/MAPK pathway. CR-1 is a newly discovered member of the EGF family of peptides that is structurally unique within this family as CR-1 lacks an A loop and possesses a truncated B loop (2, 3, 7). Since conserved amino acid residues in the A loop in conjunction with residues in the C loop are necessary for peptide binding to the EGFR receptor (37) and since the 47-mer refolded CR-1 related peptide does not directly compete with EGF for binding to the EGFR (3), this demonstrates that CR-1 cannot bind to this receptor. Nevertheless, CR-1 is able to stimulate the proliferation and differentiation of HC-11 cells[2] and the growth of several different nontransformed human mammary epithelial and breast cancer cell lines suggesting that a receptor exists for this peptide (3). In this context, the synthetic CR-1 peptide interacts with a high-affinity binding site on HC-11 mouse mammary epithelial cells and on several different human breast cancer cell lines that exhibit specificity for the CR-1 47-mer peptide since other EGF-related peptides that bind either to the EGFR or c-*erb* B-3 and c-*erb* B-4 fail to compete for binding with the labeled p47 CR-1 peptide. This suggests that the CR-1-binding site is unique. Conversely, the CR-1 peptide or recombinant GST-CR-1 fusion protein does not directly activate the tyrosine kinase of either the EGFR or other members of the type 1 receptor tyrosine kinase family either alone or in various heterodimeric combinations in mouse Ba/F3 cells (22, 23). Nevertheless, the ability of 47-mer CR-1 peptide to induce the tyrosine phosphorylation of 185- and 120-kDa proteins in HC-11 mouse mammary epithelial cells and MDA-MB-453 or SKBr-3 human breast cancer cells suggests that CR-1 binds to a potential receptor that has either an intrinsic tyrosine kinase

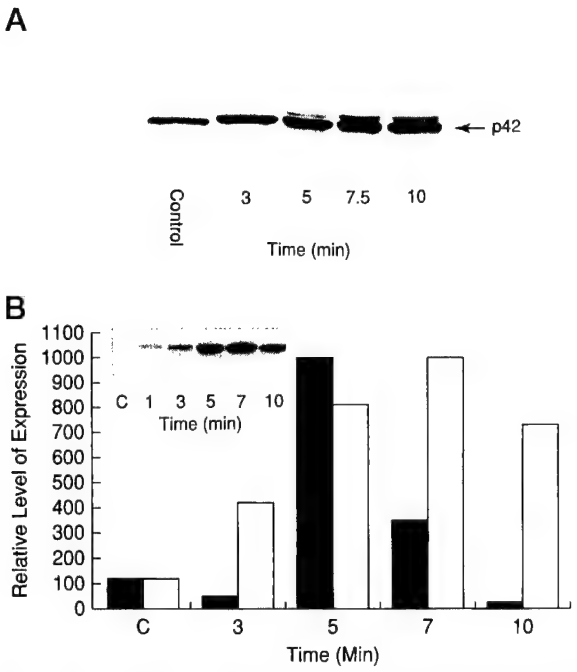

FIG. 5. **Effect of CR-1 on MAPK activation in HC-11 cells.** HC-11 cells were treated for various times with p47 CR-1 (100 ng/ml). In *A* crude cell lysates were run on 10% SDS-PAGE gels and probed with an anti-MAPK antibody (Santa Cruz) to show the hyperphosphorylated, slower migrating band of $p42^{erk\text{-}2}$. In *B*, *insert*, cell lysates were immunoprecipitated with anti-erk-2 antibody (Upstate Biotechnology) and assayed for MAPK activity by an *in vitro* kinase assay using MBP as a substrate followed by electrophoresis and autoradiography. Blots *B* in *3* and *5* were scanned densitometrically using NIH Image program 1.58 and the phosphorylation of Shc (■) and MAPK (□) relative to control are represented graphically.

activity or that associates with a tyrosine kinase. In this context, it is known that various members of the type 1 receptor tyrosine kinase family can heterodimerize following ligand binding or associate with $p60^{c\text{-}src}$. Heterodimerization might facilitate the activation of different signaling proteins thereby contributing to signal amplification and diversification in response to different type 1 receptor ligands (14, 15, 36). The present experiments do not formally exclude the possibility that the CR-1 receptor can also heterodimerize with one of these type 1 receptor tyrosine kinases or with other soluble *src*-related tyrosine kinases and that these interactions may be essential for propagation of an intracellular signal.

The present data also demonstrates that p47 CR-1 treatment of HC-11, MDA-MB-453, or SKBr-3 cells can lead to a rapid increase in tyrosine phosphorylation of the p66, p52, and p46 isoforms of Shc which can then associate with the Grb2-mSOS signaling complex. Since this is one possible mechanism by which other growth factor receptor tyrosine kinases can couple to the MEK/MAPK pathway through *ras* and *raf* (27–30, 38), these results may be functionally significant with respect to defining components in the intracellular signal transduction pathway that are activated by the CR-1 receptor. Moreover, the results demonstrate that CR-1 can activate $p42^{erk\text{-}2}$ MAPK by rapidly inducing the tyrosine phosphorylation of this MAPK isoform. Since activation of MAPK by various growth factors appears in certain cells to be obligatory for cell proliferation and/or for differentiation (16), then this same situation may also be applicable to CR-1 in its ability to stimulate growth and modulate the expression of β-casein and whey acidic protein in HC-11 cells and in primary mouse mammary epithelial cells in response to lactogenic hormones such as prolactin.[2] Identification and characterization of the CR-1 receptor following chemical cross-linking of its ligand should clarify some of these issues. In this respect, attempts to chemically cross-link the ^{125}I-p47 CR-1 peptide have been unsuccessful. However, we have recently expressed a full-length, refolded, biologically active human CR-1 protein in *Escherichia coli* and in Sf9 insect cells with a baculovirus expression vector.[3] Cross-linking of these proteins to appropriate target cells such as HC-11 should now be feasible.

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Ligands for ErbB-family receptors encoded by a neuregulin-like gene

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Neuregulins (also called ARIA[1], GGF[2], heregulin[3] or NDF[4]) are a group of polypeptide factors that arise from alternative RNA splicing of a single gene. Through their interaction with the ErbB family of receptors (ErbB2, ErbB3 and ErbB4), neuregulins help to regulate cell growth and differentiation in many tissues[5–7]. Here we report the cloning of a second neuregulin-like gene, neuregulin-2. The encoded product of the neuregulin-2 gene has a motif structure similar to that of neuregulins and an alternative splicing site in the epidermal growth factor(EGF)-like domain gives rise to two isoforms (α and β). Northern blot and *in situ* hybridization analysis of adult rat tissues indicate that expression of neuregulin-2 is highest in the cerebellum, and the expression pattern is different from that of neuregulins. Recombinant neuregulin-2β induces the tyrosine-phosphorylation of ErbB2, ErbB3 and ErbB4 in cell lines expressing all of these ErbB-family receptors. However, in cell lines with defined combinations of ErbBs, neuregulin-2β only activates those with ErbB3 and/or ErbB4, suggesting that signalling by neuregulin-2 is mediated by ErbB3 and/or ErbB4 receptors.

ErbB2, ErbB3 and ErbB4[8–10] are members of a subfamily of receptor tyrosine kinases that also includes the EGF receptor (EGFR). Signalling through ErbB family receptors is important for regulating cell proliferation and differentiation in many tissues[5–7], and deregulation of these signalling pathways is implicated in a variety of cancers[11]. Although it has been demonstrated that neuregulins can activate ErbB2/3/4 receptors through direct or indirect interaction[12,13], additional ligands for ErbB-family receptors may exist[14–16]. We used a polymerase chain reaction (PCR) based strategy to search for neuregulin-related sequences in an adult rat cerebellum complementary DNA library and have identified a new neuregulin-like gene[17], neuregulin-2.

Figure 1 shows the deduced amino-acid sequence of neuregulin-2β, derived from a composite of two overlapping cDNA clones. This composite contains an open reading frame (ORF) encoding a 754-amino-acid protein. Sequence analysis revealed four structural motifs: a putative signal sequence, a C2-type immunoglobulin-like (Ig-like) domain[18], an EGF-like domain (residues 252–297) with its six characteristic cysteines[19], and a putative transmembrane domain (which separates the whole sequence into a 315-residue extracellular domain and a 414-residue cytoplasmic domain). Another neuregulin-2 cDNA clone, with an extra 77-base-pair (bp) exon inserted between the fourth and fifth cysteine residues of the EGF-like domain, encodes an alternatively spliced variant of neuregulin-2 with a different EGF-like domain (see Supplementary Information). This neuregulin-2 isoform also lacks a transmembrane domain, because the insertion of the extra exon causes a frameshift in the downstream sequence and the termination of the ORF 33 amino acids downstream of the EGF-like domain. Neuregulin-2 molecules having two variant EGF-like domains are termed neuregulin-2α and neuregulin-2β, respectively. The neuregulin gene also has a similar alternative splicing site that gives rise to the α- and β-subtypes of neuregulins[2,3,20], although neuregulin-2α and neuregulin-2β are about equally distant from neuregulin-α or from neuregulin-β. Moreover, there is another alternative splicing site in the cytoplasmic domain of neuregulin-2 in other neuregulin-2 cDNA clones (data not shown), corresponding to the a/b/c-tail splicing site in the neuregulin gene[20]. Therefore, neuregulin-2 and neuregulin not only have similar sequences, they also have similar gene structures.

A protein-database search revealed that the neuregulin-2 proteins are most similar to neuregulins (and to heregulin-β1 among the isoforms of the neuregulins). Overall neuregulin-2β shares 45% identity with heregulin-β1 (ref. 3) and 40% with GGFII (ref. 2). Apart from the N terminus of neuregulin-2β, the similarity between neuregulin-2β and heregulin-β1 extends through their entire sequence (Fig. 2). On the other hand, the N terminus of neuregulin-2β has significant identity to that of GGFII (43%) (Fig. 2). The

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1   MRRDPAPGFSMLLFGVSLACYSPSLKSVQDQAYKAPVVVEGKVQGLAPAGGSSSNSTREP   60
61  PASGRVALVKVLDKWPLRSGGLQREQVISVGSCAPLERNQRYIFFLEPTEQPLVFKTAFA   120
121 PVDPNGKNIKKEVGKILCTDCATRPKLKKMKSQTGEVGEKQSLKCEAAAGNPQPSYRWFK   180
181 DGKELNRSRDIRIKYGNGRKNSRLQFNKVKVEDAGEYVCEAENILGKDTVRGRLHVNSVS   240
241 TTLSSWSGHAHKCNETAKSYCVNGGVCYYIEGINQLSCKCPVGYTGDRCQQFAMVNFSKH   300
301 LGFELKEAEELYQKRVLTITGICVALLVVGIVCVVAYCKTKKQRRQMHHHLRQNMCPAHQ   360
361 NRSLANGPSHPRLDPEEIQMADYISKNVPATDHVIRREAETTFSGSHSCSPSHHCSTATP   420
421 TSSHRHESHTWSLERSESLTSDSQSGIMLSSVGTSKCNSPACVEARARRAAAYSQEERRR   480
481 AAMPPYHDSIDSLRDSPHSERYVSALTTPARLSPVDFHYSLATQVPTFEITSPNSAHAVS   540
541 LPPAAPISYRLAEQQPLLRHPAPPGPGPGPGADMQRSYDSYYYPAAGPGPRRGACALGGS   600
601 LGSLPASPFHIPEDDEYETTQECAPPPPPRPRTRGASRRTSAGPRRWRRSRLNGLAAQRA   660
661 RAARDSLSLSSGSGCGSASASDDDADDADGALAAESTPFLGLRAAHDALRSDSPPLCPAA   720
721 DSRTYYSLDSHSTRASSRHSRGPPTRAKQDSGPL  754
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Figure 1 Deduced amino-acid sequence of rat neuregulin-2β. Arrowed underline marks the putative signal sequence. The immunoglobulin-like domain is outlined by a dashed box. Solid frame surrounds the EGF-like domain; the six cysteines characteristic of this domain are indicated by asterisks. Potential *N*-glycosylation sites are marked with arrowheads. The putative transmembrane region is underlined. An arrow points to the potential proteolytic site.

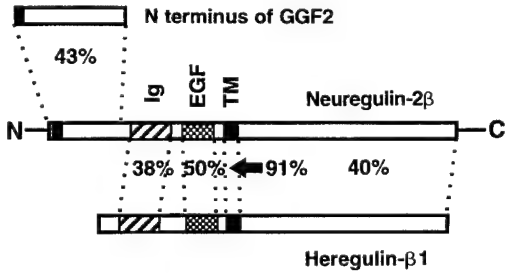

Figure 2 The motif structure of the neuregulin-2β and its similarity to selected members of neuregulins. The percentage similarity is calculated from the amino-acid sequence alignment of neuregulin-2β, heregulin-β1 (human)[3] and the N terminus of GGFII (human)[2]. Black boxes, potential signal sequences; Ig, immunoglobulin-like domains; EGF, EGF-like domains; TM, transmembrane domains.

most similar region between neuregulin-2β and heregulin-β1 is the transmembrane domain (91% identity) and adjacent sequence. Highly conserved regions also exist in the cytoplasmic tails of neuregulin-2β and heregulin-β1, indicating that the cytoplasmic domains may be important biologically. Relatively high conservation between neuregulin cytoplasmic tails from distant vertebrate species has been noted before[1]. As the EGF-like domain of neuregulins is sufficient for receptor binding and for stimulating cellular responses[3], we compared the EGF-like domain of neuregulin-2 molecules with other EGF-like motifs. Among the known EGF-like motifs, the EGF-like domain of neuregulin-2 is most similar to that of the neuregulins (48% identity between terminal cysteines in the case of heregulin-β1). Second to neuregulins is the rat epidermal growth factor, with 43% identity between terminal cysteines.

To determine the size and tissue distribution of neuregulin-2 mRNAs, northern blot hybridization with poly(A)$^+$ RNA was carried out using a probe spanning the EGF-like domain plus the immunoglobulin-like domain (Fig. 3A). Among the adult rat tissues examined, neuregulin-2 transcripts were most abundant in neural tissues (brain and spinal cord) and lung. A separate experiment with total RNA samples shows that the cerebellum has the highest concentration of neuregulin-2 transcripts compared to other parts of the brain and other adult tissues (data not shown). Three bands were seen in brain samples (Fig. 3A): a prominent band of 3 kilobases (kb), and two additional bands of 3.8 and 6 kb. Only the 3- and 3.8-kb transcripts were detected in spinal cord and lung samples. This pattern of three principal transcripts has also been found for the neuregulin gene, but at the sensitivity of the northern blot, the tissue distribution of neuregulin-2 transcripts in adult rat seems to be more restricted than that of neuregulins[3,4].

We also characterized neuregulin-2 expression by *in situ* hybridization with several probes. In adult rat brain sections, the highest expression was detected in the cerebellum (in the Purkinje cell layer and the granule cell layer) and in the dentate gyrus of the hippocampus (Fig. 3B). Labelled cells were also found in the olfactory bulb (data not shown). This expression pattern seems to be distinct from that of neuregulins, because no hybridization signal for neuregulins is observed in Purkinje cells and very little in the granule cell layer[21]. We investigated the expression pattern of

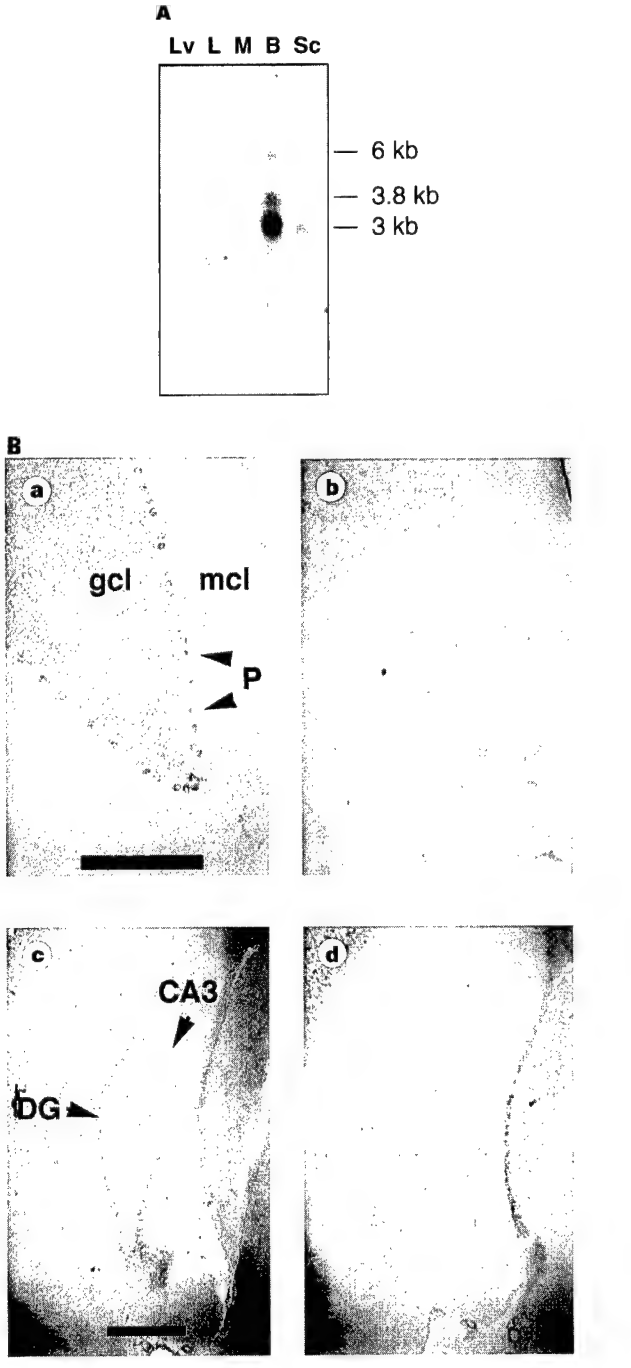

Figure 3 Expression of neuregulin-2 transcripts in adult rat tissues. **A**, Northern blot analysis using poly(A)$^+$ RNA samples; approximately 2 μg poly(A)$^+$ RNA was loaded in each lane. The three bands detected (3, 3.8, 6 kb) are indicated. Lv, liver; L, lung; M, skeletal muscle; B, brain; Sc, spinal cord; **B**, *In situ* hybridization of adult rat brain parasagittal sections with a digoxingenin-labelled cRNA probe spanning the EGF-like and Ig domains. **a**, Neuregulin-2 transcripts were detected in Purkinje cells (P) and in the granule cell layer (gcl) in the cerebellum, but not in the molecular cell layer (mcl); scale bar, 0.4 mm. **b**, Adjacent section hybridized with a sense control probe. **c**, In the hippocampus, neuregulin-2 transcripts were only detected in the dentate gyrus (DG) but not in the CA1–CA3 area; scale bar, 0.8 mm. **d**, Adjacent section hybridized with the sense control probe.

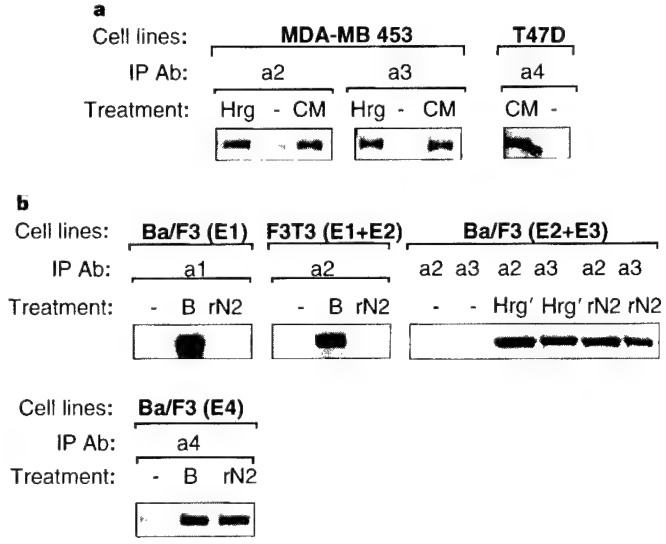

Figure 4 Recombinant neuregulin-2β protein induces tyrosine-phosphorylation of ErbB-family receptors through ErbB3 and ErbB4. **a**, Neuregulin-2β induces tyrosine phosphorylation of ErbB2, ErbB3 and ErbB4 in MDA-MB 453 and T47D cell lines. **b**, Neuregulin-2β signalling through ErbB3 and ErbB4 receptors. rNRG-2β was tested on cell lines transfected with defined ErbB-family receptors. Only cells with ErbB3 and/or ErbB4 receptors were activated. E1, EGF receptor; E2, ErbB2; E3, ErbB3; E4, ErbB4. Immunoprecipitating antibodies (IP Ab): a1, anti-EGF receptor; a2, anti-ErbB2; a3, anti-ErbB3; a4, anti-ErbB4. B, betacellulin; Hrg', heregulin-β1 EGF-like domain; rN2, rNRG-2β (EGF-like domain of neuregulin-2β); CM, neuregulin-2β-conditioned medium; −, negative control.

neuregulin-2 during embryogenesis by whole-mount *in situ* hybridization with the same probes. We did not detect any signal in E9.5–E10 mouse embryos (data not shown), indicating that none or very little neuregulin-2 is expressed in embryos at those developmental stages. Therefore neuroregulin-2 is unlikely to be the 'missing ligand' for ErbB4 receptors[15].

The structural similarity between neuregulin-2 and neuregulins suggests that the neuregulin-2 proteins may also function as ligands for ErbB family receptors. To test this, we expressed a large portion of neuregulin-2β (including all of the extracellular domain and part of cytoplasmic domain) in CHO cells. As the sequences around the putative proteolysis sites are highly conserved between neuregulin-2 and neuregulins, a soluble form of neuregulin-2β protein should be released from its precursors to the culture medium, as in the case of neuregulins[1–4]. We treated cells expressing ErbB family receptors (MDA-MB453 and T47D breast cancer cell lines)[22,23] with conditioned medium from stably transfected CHO cells. As shown in Fig. 4a, ErbB2, ErbB3 and ErbB4 receptors were activated by neuregulin-2β-conditioned medium. But as ErbB family receptors can form ligand-induced heterodimers, the activation of ErbB2/3/4 receptors could be due to direct or indirect interaction with neuregulin-2β. We also expressed the EGF-like domain (amino acids 240–305; Fig. 1) of neuregulin-2β in bacteria and produced a refolded neuregulin-2β protein fragment (rNRG-2β) from inclusion bodies. rNRG-2β can activate ErbB-family receptors in our breast-tumour cell lines (data not shown), suggesting that like neuregulins, the EGF-like domain is the functional domain for activating ErbB-family receptors.

To determine which of the ErbB family receptors is involved in neuregulin-2β signalling, we tested rNRG-2β on cell lines expressing defined combinations of ErbB receptors. We did not detect rNRG-2β activation of EGF receptors in the Ba/F3 (EGFR) cell line or of ErbB2 receptor in the Fischer rat 3T3 cell line (Fig. 4b), whereas our positive control, betacellulin[24], stimulated these receptors. On the other hand, rNRG-2β stimulated ErbB4 receptor in the Ba/F3 (ErbB4) cell line, as well as ErbB2 and ErbB3 receptors in the Ba/F3 (ErbB2 + ErbB3) cell line. These results indicate that neuregulin-2β signalling results from direct interaction with ErbB3 and/or ErbB4 receptors.

We have shown that the neuregulin-2 gene, which has structural similarity to the neuregulin gene, encodes new ligands for the ErbB3 and ErbB4 receptors. The distinct expression pattern of neuregulin-2 suggests that these proteins have specific biological functions. It will be necessary to compare neuregulin-2 with neuregulins and other ligands for ErbB-family receptors, including the temporal and spatial regulation of their expression, in order to understand the function of this multiligand/multireceptor signalling network.

Methods

Cloning of neuregulin-2 cDNAs. Two pools of degenerate oligonucleotides were synthesized based on two conserved regions of the neuregulin sequences, one in the immunoglobulin-like domain and the other in the EGF-like domain. Phages from an adult rat cerebellum cDNA library (gift from D. Zhao) were used as templates for PCR. Two steps were used to reduce neuregulin sequences and select neuregulin-related sequences. First, PCR products were digested with *Bcl*I and separated by agarose gel electrophoresis, because there is a *Bcl*I site in rat neuregulin cDNA[4]. DNA fragments of expected sizes were isolated from the agarose gel and reamplified with the same primers. Final PCR products were subcloned into pBlueScriptII vector (Stratagene). Second, individual clones were hybridized with a neuregulin probe under low-stringency conditions and positive clones were sequenced. We identified one clone, n9, which has significant homology to neuregulins. ^{32}P-labelled probes from the n9 insert were used to screen the cDNA library (~500,000 clones) and several positive clones were identified. The insert of each clone was sequenced in both directions and analysed.

Northern blot and *in situ* hybridization. Poly(A)$^+$ RNA was purified from tissues by using a FastTrack kit (Invitrogen). RNA samples were separated on agarose gels and transferred to nylon filters by standard procedures. Filters were hybridized with ^{32}P-labelled probes under high-stringency conditions. A probe was generated by random priming of a fragment of neuregulin-2 cDNAs spanning the EGF-like plus the Ig-like domains. The highly conserved transmembrane domain and adjacent sequence were excluded. The probe would hybridize to both neuregulin-2α and neuregulin-2β transcripts. *In situ* hybridization was done essentially as described[25]. We used a digoxigenin-labelled cRNA probe spanning the EGF-like plus the Ig-like domains. Several other probes derived from different parts of neuregulin-2 cDNAs (that is, the EGF-like domain only, the Ig domain only) also gave essentially the same hybridization pattern.

Expression of recombinant neuregulin-2 proteins. The insert of a partial neuregulin-2β cDNA clone was subcloned into the pRc/CMV expression vector (Invitrogen) and stably transfected into CHO cells. Serum-free conditioned medium was collected. Negative control media were conditioned medium from CHO cells or from CHO cells transfected with an unrelated gene. For expression of rNRG-2β in *E. coli*, the EGF-like domain of neuregulin-2β (residues 240–305) was subcloned into pQE32 expression vector with an N terminus 6 × histidine tag (Qiagen). Protocols for solubilization and refolding of proteins from inclusion bodies were essentially as described[24], except that refolded proteins were not purified further. The final rNRG-2β protein concentration is ~500 μg ml^{-1}.

Tyrosine-phosphorylation assay. MDA-MB 453 and T47D cells were starved in serum-free medium for 2–6 h before addition of neuregulin-2β conditioned medium, negative control medium, or heregulin-β1 (extracellular portion, 20 ng ml^{-1}; from S. J. Burden). After 5–10 min at room temperature, cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1 SDS, 1 mM sodium orthovanadate, 50 μg ml^{-1} aprotinin, 0.5 mM PMSF), immunoprecipitated with rabbit antibodies (Santa Cruz Biotechnology) specific for ErbB2 (C18), ErbB3 (C17) or ErbB4 (C18). Immunoprecipitated proteins were collected on protein A–Sepharose beads, analysed by western blotting with an anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology). Antibody binding was detected by enhanced chemiluminescence (Amersham Life Science). The recombinant Ba/F3 cell lines expressing ErbB family receptors have been described, as have protocols for stimulating and analysing ErbB-family receptor tyrosine-phosphorylation[24,26,27]. EGFR and ErbB2 expression in Fischer rat 3T3 (F3T3) was described[28]. Human recombinant betacellulin (R&D Systems) was used at 200 ng ml^{-1}. Chemically synthesized heregulin-β1 65-mer[29] was used at 94 ng ml^{-1}. Although we can detect the activity of rNRG-2β at a dilution of 1 : 50,000, it was routinely used at a dilution of 1 : 100 to ensure saturated receptor phosphorylation.

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Activation of ErbB4 by the Bifunctional Epidermal Growth Factor Family Hormone Epiregulin Is Regulated by ErbB2*

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Epiregulin (EPR) is a recently described member of the epidermal growth factor (EGF) family of peptide growth factors. The ever expanding size of the EGF family has made distinguishing the activities of these hormones paramount. We show here that EPR activates two members of the ErbB family of receptor tyrosine kinases, epidermal growth factor receptor (EGFR) and ErbB4. Therefore by these criteria, EPR is qualitatively similar to another EGF family hormone, betacellulin (BTC). Yet, here we also demonstrate quantitative differences between EPR and BTC. EPR stimulates higher levels of EGFR phosphorylation than does BTC, whereas BTC stimulates higher levels of ErbB4 phosphorylation than does EPR. Moreover, the EPR and BTC dose response curves show that although EGFR is more sensitive to EPR than is ErbB4, ErbB4 is more sensitive to BTC than is EGFR. Finally, ErbB2, which is not activated by EPR when expressed on its own, increases the sensitivity of ErbB4 for activation by EPR. Therefore, these results establish that EPR exhibits novel activities and modes of regulation, which may have significant implications for EPR function *in vivo*.

The continuing discovery of novel members of the epidermal growth factor (EGF)[1] family of peptide growth factors has led to an increased appreciation of the functional differences among these hormones, as well as a realization of the complex hormone-receptor interactions fostered by these peptides. EGF, transforming growth factor α (TGF-α), and amphiregulin all bind exclusively to the EGF receptor (EGFR). Yet, these hormones can also activate *in trans* (transmodulate) the other three ErbB family receptors (Neu/ErbB2/Her2, ErbB3/Her3, ErbB4/Her4) through ligand-induced receptor heterodimerization with the EGFR (1–8). Other EGF family hormones bind multiple receptors. Neuregulin (NRG) and neuregulin2 (NRG2) bind ErbB3 and ErbB4 and transmodulate EGFR and ErbB2 (9–16). Betacellulin (BTC) combines some of the properties of EGF and NRG by activating EGFR and ErbB4 (7).

EPR was initially purified from the conditioned medium of a tumorigenic clone of NIH3T3 fibroblasts. It competes with EGF for binding to A431 cells, which overexpress EGFR, suggesting that EPR is a ligand for EGFR (17). Since at least one of the EGF family hormones that activates EGFR also activates ErbB4, we wished to evaluate EPR function in a set of cell lines expressing all four ErbB family receptors, both singly and in every pairwise combination.

We demonstrate here that EPR activates not only EGFR, but ErbB4 as well. However, the dose-response curves for BTC and EPR in a cell line expressing both EGFR and ErbB4 are markedly different. Whereas ErbB4 is more responsive to BTC than is EGFR, ErbB4 is less responsive to EPR than is EGFR. Moreover, ErbB2 expression increases saturated ErbB4 phosphorylation in response to EPR and dramatically enhances the sensitivity of ErbB4 for activation by EPR as well. In this respect EPR resembles NRG, which displays a low affinity for ErbB3 that increases in cells where ErbB2 is co-expressed (12).

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture—BaF3 is an immortal mouse lymphoblastoid cell line (31). BaF3-derived cell lines expressing combinations of ErbB family receptors have been described previously (14). The ranked order of receptor expression in the double recombinant BaF3 cell lines is as follows. For EGFR expression, BaF3/EGFR+ErbB4 is higher than BaF3/EGFR+ErbB2, which is higher than BaF3/EGFR+ErbB3. For ErbB2 expression, BaF3/ErbB2+ErbB4 is equivalent to BaF3/EGFR+ErbB2, both of which are markedly higher than BaF3/ErbB2+ErbB3. The levels of ErbB3 expression are similiar in the BaF3/EGFR+ErbB3, BaF3/ErbB2+ErbB3, and BaF3/ErbB3+ErbB4 cell lines. The levels of ErbB4 expression are similiar in the BaF3/EGFR+ErbB4, BaF3/Neu+ErbB4, and BaF3/ErbB3+ErbB4 cell lines (7, 14).

CEM is an immortal human T-lymphoblastoid cell line that does not endogenously express EGF receptor, ErbB2, ErbB3, or ErbB4. CEM-derived cell lines expressing ErbB4 or ErbB2 and ErbB4 have been described previously (10). Cell culture conditions were as described (10, 14).

Growth Factors—Recombinant human EPR was produced in *Bacillus brevis*.[2] Recombinant NRGβ was the generous gift of Kerry Russell and Jeffrey Bender (Yale University). We are grateful to Jim Moyer, Brad Guarino, and Glenn Andrews (Pfizer Central Research, Groton, CT) for synthetic NRGβ (32). Recombinant BTC and NRGβ were purchased from R & D Systems (Minneapolis, MN), whereas recom-

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[1] The abbreviations used are: EGF, epidermal growth factor; TGF-α, transforming growth factor α; EGFR, EGF receptor; NRG, neuregulin; BTC, betacellulin; IL3, interleukin 3; PAGE, polyacrylamide gel electrophoresis.

[2] T. Nakazawa *et al.*, in preparation.

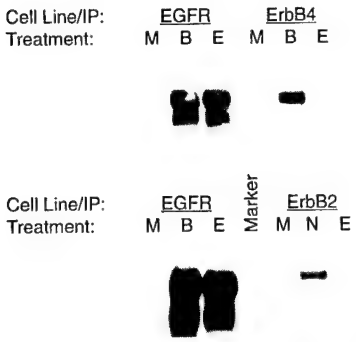

FIG. 1. **EPR stimulation of receptor phosphorylation in Ba/F3 cells expressing a single ErbB family receptor.** BaF3/EGFR, BaF3/ErbB2, and BaF3/ErbB4 cells (14) were stimulated with 1000 ng/ml EPR (*E*), 100 ng/ml BTC (*B*), 100 ng/ml recombinant NRGβ (*N*), or were mock stimulated with phosphate-buffered saline (*M*) as described previously (7, 14). ErbB family receptors were immunoprecipitated from lysed cells using specific antireceptor antibodies and separated by SDS-PAGE as described previously (7, 14). Tyrosine-phosphorylated ErbB family receptors were detected and visualized by immunoblotting using the 4G10 monoclonal antiphosphotyrosine antibody as described previously (7, 14).

binant TGF-α was purchased from Collaborative Biomedical Products (Bedford, MA).

Stimulation and Analysis of Receptor Phosphorylation—The conditions for stimulation of ErbB family receptor tyrosine phosphorylation have been described previously (7, 14). The analysis of ErbB family receptor tyrosine phosphorylation by immunoprecipitation and antiphosphotyrosine immunoblotting has been described previously (7, 14). Immunoprecipitating antireceptor antibodies were anti-EGFR mouse monoclonal antibody 528 (33), anti-ErbB2 mouse monoclonal antibody TA-1 (OP-39, Calbiochem), anti-ErbB3 rabbit polyclonal antiserum SC-285 (Santa Cruz Biotechnology), and anti-ErbB4 rabbit polyclonal antiserum SC-283 (Santa Cruz Biotechnology). Specificity of antireceptor antibodies has been verified by testing for cross-reactivity (data not shown).

Immunoblot autoradiographs were digitized using a Hewlett-Packard 3p flatbed scanner set for 600 dpi resolution and controlled by Hewlett-Packard Deskscan II for Macintosh software. Images were cropped using Adobe Photoshop, and the band intensity was quantified using NIH Image software. Net receptor activation was calculated by subtracting the amount of tyrosine phosphorylation observed in samples from mock-stimulated cells.

RESULTS

EPR Activates EGFR—We first sought to identify which ErbB family receptors are activated by EPR when the receptors are expressed individually. We previously developed a panel of cell lines based on the mouse BaF3 hematopoietic cell line that expresses the four ErbB family receptors, both singly and in every pairwise combination. Hence, we incubated the BaF3 cell lines ectopically expressing EGFR, ErbB2, or ErbB4 with EPR. EPR, like BTC, stimulated EGFR tyrosine phosphorylation, consistent with published results suggesting that EPR binds EGFR (17) (Fig. 1, *EGFR panel*; compare *lanes E* and *B* with *M*). However, EPR did not stimulate phosphorylation of ErbB2 or ErbB4 (Fig. 1, *ErbB2* and *ErbB4 panels*; compare *lanes E* and *M*). In contrast, the positive control NRGβ stimulated ErbB2 tyrosine phosphorylation and BTC stimulated ErbB4 phosphorylation. The ErbB2 phosphorylation observed in BaF3/ErbB2 cells stimulated with NRG is the result of transmodulation of ErbB2 by the NRG receptor ErbB3, which is endogenously expressed at low levels in BaF3 cells (14). Neither EPR nor any of the other EGF family ligands tested to date stimulated ErbB3 tyrosine phosphorylation in BaF3 cells expressing only ErbB3 (data not shown) (7, 8, 14). However, since ErbB3 lacks tyrosine kinase activity (18), these experiments do not rule out EPR binding to ErbB3.

Since EPR activates EGFR, we next determined whether EPR activates the other three ErbB family receptors *in trans*

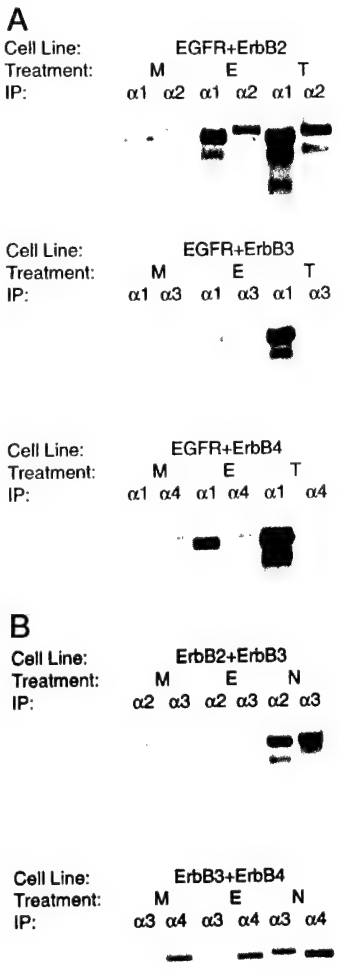

FIG. 2. ***A* and *B*, EPR stimulation of receptor phosphorylation in BaF3 cells expressing combinations of ErbB family receptors.** BaF3/EGFR+ErbB2, BaF3/EGFR+ErbB3, BaF3/EGFR+ErbB4, BaF3/ErbB2+ErbB3, and BaF3/ErbB3+ErbB4 cells (14) were stimulated with 200 ng/ml EPR (*E*), 200 ng/ml TGF-α (*T*), 188 ng/ml synthetic NRGβ (*N*), or were mock stimulated with phosphate-buffered saline (*M*) as described previously (7, 8, 14). EGFR (α*1*), ErbB2 (α*2*), ErbB3 (α*3*), or ErbB4 (α*4*) was immunoprecipitated from lysed cells using specific antireceptor antibodies and separated by SDS-PAGE as described previously (7, 14). Tyrosine-phosphorylated ErbB family receptors were detected and visualized by immunoblotting using the 4G10 monoclonal antiphosphotyrosine antibody as described previously (7, 14).

via EGFR. A panel of BaF3 cell lines ectopically expressing EGFR together with one of the other three ErbB family receptors was stimulated with EPR. EPR activated the EGFR in all three cell lines (Fig. 2*A*; compare *E* α*1 lanes* with the *M* α*1 lanes*). Both EPR (*E lanes*) and the positive control TGF-α (*T lanes*) strongly activated ErbB2 in the cell line co-expressing EGFR+ErbB2 (Fig. 2*A*, *EGFR+ErbB2 panel*; α*2 lanes*). In contrast, neither EPR nor TGF-α activated ErbB3 or ErbB4 (Fig. 2*A*, *EGFR+ErbB3* and *EGFR+ErbB4 panels*; α*3* or α*4 lanes*). This is consistent with the conclusion that ErbB2 is a preferential target for transmodulation by the EGFR compared with the other ErbB family receptors (7, 8, 19–21). However, higher concentrations of EPR than those used for these experiments did stimulate ErbB4 phosphorylation in the EGFR+ErbB4 cell line (see below; Fig. 6*A*).

Since ErbB3 lacks functional kinase activity, EGF family hormones can activate ErbB3 only in the presence of another ErbB family receptor, particularly ErbB2, which permits the highest levels of ErbB3 phosphorylation by NRG (11, 12, 14).

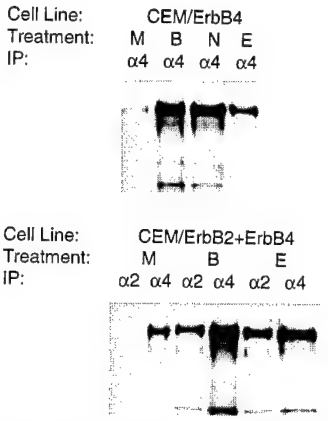

FIG. 3. **EPR stimulation of receptor phosphorylation in CEM cells expressing either ErbB4 alone or both ErbB2 and ErbB4.** CEM/ErbB4 and CEM/ErbB2+ErbB4 cells (10) were stimulated with 100 ng/ml BTC (*B*), 100 ng/ml recombinant NRGβ (*N*), 1000 ng/ml EPR (*E*), or were mock stimulated with phosphate-buffered saline (*M*) as described previously (14). ErbB2 ($\alpha 2$) or ErbB4 ($\alpha 4$) was immunoprecipitated from lysed cells using specific antireceptor antibodies and separated by SDS-PAGE as described previously (7, 14). Tyrosine-phosphorylated ErbB family receptors were detected and visualized by immunoblotting using the 4G10 monoclonal antiphosphotyrosine antibody as described previously (7, 14).

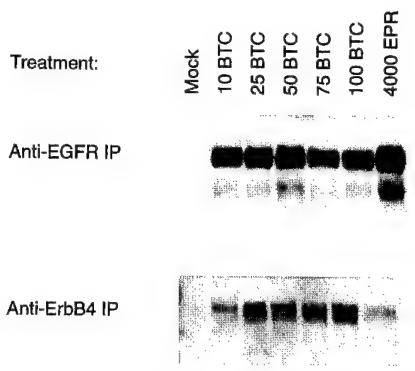

FIG. 4. **Stimulation of receptor phosphorylation in BaF3 cells expressing both EGFR and ErbB4 by EPR and increasing doses of BTC.** BaF3/EGFR+ErbB4 cells (14) were stimulated with 10, 25, 50, 75, or 100 ng/ml BTC, 4000 ng/ml EPR, or mock stimulated with phosphate-buffered saline as described previously (7, 8, 14). EGFR or ErbB4 was immunoprecipitated from lysed cells using specific antireceptor antibodies and separated by SDS-PAGE as described previously (7, 14). Tyrosine-phosphorylated ErbB family receptors were detected and visualized by immunoblotting using the 4G10 monoclonal antiphosphotyrosine antibody as described previously (7, 14).

Therefore, BaF3 cells expressing both ErbB2 and ErbB3 or both ErbB3 and ErbB4 were stimulated with EPR to determine if ErbB3 is a receptor for EPR. In the ErbB2+ErbB3 cell line, the positive control NRGβ activated both receptors (Fig. 2*B*, *ErbB2+ErbB3 panel*, compare *N lanes* with *M lanes*), whereas in the ErbB3+ErbB4 cell line NRGβ stimulated a marked increase in ErbB3 phosphorylation and a modest increase in ErbB4 phosphorylation (Fig. 2*B*, *ErbB3+ErbB4* panel, compare *N lanes* with *M lanes*). In contrast, EPR did not stimulate receptor phosphorylation in either of these cell lines, suggesting that ErbB3 is not a receptor for EPR (Fig. 2*B*; compare *E lanes* with *M lanes*).

EPR Activates ErbB4 in CEM Cells—BTC activates both ErbB4 and EGFR when expressed individually (7). We tested whether EPR behaves like BTC and also activates ErbB4 when expressed alone using derivatives of the CEM human T-lymphoblastoid cell line that ectopically expresses ErbB4 alone or both ErbB2 and ErbB4 (10). EPR activated ErbB4 in CEM cells expressing ErbB4 alone and both receptors in CEM cells expressing ErbB2 and ErbB4 together (Fig. 3; compare *E lanes* with *M lanes*). In experiments done in parallel using identical growth factor concentrations, EPR did not activate ErbB4 in BaF3 cells expressing ErbB4 alone (also see Fig. 1) but stimulated ErbB2 and ErbB4 phosphorylation in BaF3 cells expressing both ErbB2 and ErbB4 (data not shown). It is unclear why EPR failed to activate ErbB4 in the BaF3 cells expressing ErbB4 alone. Nonetheless, because EPR activates EGFR as well as ErbB4, EPR resembles BTC, which also activates these receptors (7).

EPR Stimulates EGFR More Than ErbB4 and EGFR Is More Sensitive Than ErbB4 to EPR—Since BTC and EPR can activate EGFR and ErbB4 when the receptors are expressed individually, we measured BTC and EPR stimulation of EGFR and ErbB4 phosphorylation in BaF3 cells that express both receptors together (Fig. 4). BTC stimulated saturated levels of EGFR phosphorylation at a concentration of 10 ng/ml, whereas BTC stimulated saturated levels of ErbB4 phosphorylation at a concentration of 25 ng/ml. Therefore, in subsequent experiments we have assumed that 100 ng/ml BTC stimulates saturated levels of EGFR and ErbB4 phosphorylation.

At a concentration of 4000 ng/ml, EPR stimulates slightly greater levels of EGFR phosphorylation than does BTC (Fig. 4). In contrast, 4000 ng/ml EPR stimulates much lower levels of ErbB4 phosphorylation than does BTC (Fig. 4). This suggests that EPR stimulates EGFR more than ErbB4 and that EGFR is more sensitive than ErbB4 to EPR. We investigated this possibility by comparing EGFR and ErbB4 phosphorylation in BaF3/EGFR and CEM/ErbB4 cells stimulated with increasing concentrations of EPR (Fig. 5*A*). At saturation, EPR stimulated a slightly higher level of EGFR phosphorylation (1.7-fold) than did BTC, whereas EPR stimulated a much lower level of ErbB4 phosphorylation (0.3-fold) than did BTC (Fig. 5*C*). We next compared the dose sensitivity of EGFR and ErbB4 to EPR stimulation by plotting receptor phosphorylation relative to the maximal amounts of receptor phosphorylation stimulated by EPR (Fig. 5*D*) to identify the EPR concentrations required for half-maximal receptor phosphorylation (Table I). Half-maximal EGFR phosphorylation occurred at an EPR concentration of approximately 380 ng/ml (Fig. 5*D*; Table I). In contrast, half-maximal ErbB4 phosphorylation required about a 4-fold higher concentration of EPR with half-maximal activation occurring at an EPR concentration of approximately 1790 ng/ml (Fig. 5*D*; Table I).

ErbB2 Expression Increases ErbB4 Activation by EPR and Sensitivity to EPR—The affinity of NRG for cells expressing ErbB3 is greater when these cells also express ErbB2 (12). Hence, we evaluated the possibility that ErbB2 modulates ErbB4 activation by EPR by stimulating CEM/ErbB4 and CEM/ErbB2+ErbB4 cells with increasing concentrations of EPR (Fig. 5, *A* and *B*). Relative to the BTC positive control, EPR stimulated 2-fold higher levels of ErbB4 phosphorylation in the ErbB2+ErbB4 cell line than in cells expressing ErbB4 alone (Fig. 5*C*). Therefore, ErbB2, which is not activated by EPR when expressed by itself (Fig. 1), doubles the magnitude of ErbB4 activation by EPR at saturation. We next examined the effects of ErbB2 expression on the sensitivity of ErbB4 to EPR (Fig. 5*D*). Half-maximal ErbB4 phosphorylation occurred at an EPR concentration of approximately 1790 ng/ml in the cell line expressing ErbB4 alone (Fig. 5*D*; Table I), but occurred at an EPR concentration of approximately 630 ng/ml (Fig. 5*D*; Table I) in the ErbB2+ErbB4 cell line. This shift in the EPR dose-response curve in the ErbB2+ErbB4 cell line compared with the ErbB4 cell line suggests that ErbB2 expression increases the affinity of ErbB4 for EPR and implies that ErbB2-ErbB4 heterodimers have a higher affinity for EPR than do ErbB4-

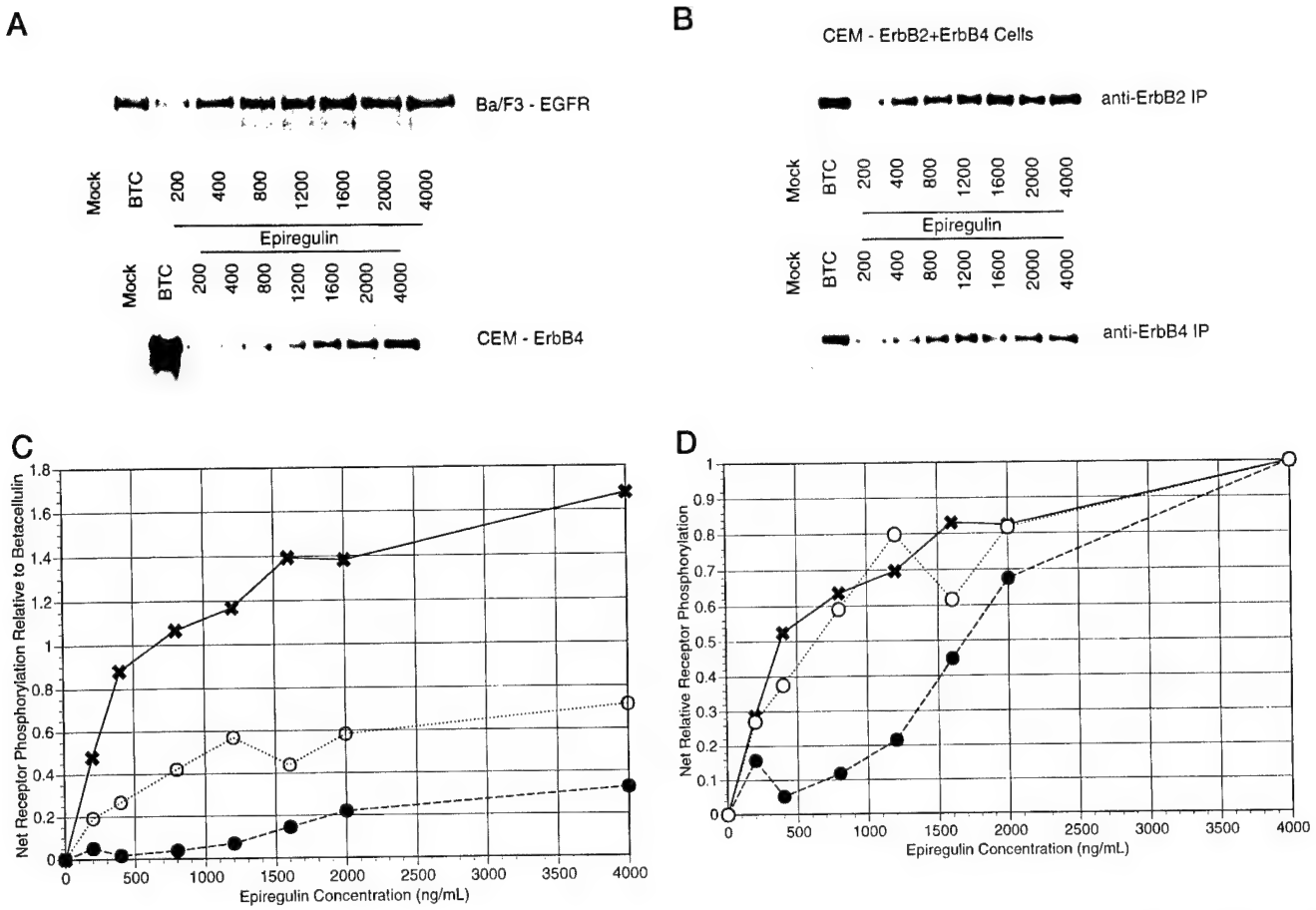

FIG. 5. **EPR dose response in BaF3 cells expressing EGFR or in CEM cells expressing ErbB4 alone or both ErbB2 and ErbB4.** *A* and *B*, BaF3/EGFR, CEM/ErbB4, or CEM/ErbB2+ErbB4 cells were stimulated with 100 ng/ml BTC (*BTC*), increasing concentrations of epiregulin as indicated or were mock stimulated with phosphate-buffered saline (*Mock*) as described previously (7, 14). EGFR, ErbB2, or ErbB4 was immunoprecipitated as indicated or appropriate using specific antireceptor antibodies and separated by SDS-PAGE as described previously (14). Tyrosine-phosphorylated ErbB family receptors were detected and visualized by immunoblotting using the 4G10 monoclonal antiphosphotyrosine antibody as described previously (7, 14). *C* and *D*, antiphosphotyrosine immunoblot images were scanned on a Hewlett-Packard ScanJet 3p flatbed scanner set for 600 dpi optical resolution. Images were cropped using Adobe Photoshop and receptor tyrosine phosphorylation was quantified using NIH Image. Net receptor tyrosine phosphorylation was calculated by subtracting the receptor tyrosine phosphorylation exhibited by mock stimulated cells. Tyrosine phosphorylation was either expressed relative to the tyrosine phosphorylation stimulated by 100 ng/ml BTC (*C*) or relative to the maximal receptor tyrosine phosphorylation stimulated by EPR (*D*). ✖, EGFR; ●, ErbB4; ○, 2+4 Anti-4.

TABLE I
Relative sensitivities of EGFR and ErbB4 to EPR and BTC
Data are adapted from Figs. 5, *A–D* and 6, *A–C*.

Cell line & receptor	EPR yielding half-maximal receptor activation	BTC yielding half-maximal receptor activation
BaF3/EGFR	380 ng/ml	NT[a]
CEM/ErbB4	1790 ng/ml	NT
BaF3/EGFR+ErbB4		
EGFR	320 ng/ml	35 ng/ml
ErbB4	790 ng/ml	5 ng/ml
CEM/ErbB2+ErbB4		
ErbB2	400 ng/ml	NT
ErbB4	630 ng/ml	NT

[a] NT, not tested.

ErbB4 homodimers.

The EPR and BTC Dose-Response Curves Are Different in Cells Expressing EGFR and ErbB4—EPR resembles BTC in its ability to activate either EGFR or ErbB4 when expressed individually (7). Yet, at saturation EPR stimulated almost 2-fold more EGFR phosphorylation than BTC, whereas BTC activated about 3-fold more ErbB4 phosphorylation than did EPR (Fig. 5, *A* and *C*). This suggested that BTC and EPR are functionally distinct. Hence, we compared EGFR and ErbB4 phosphorylation following stimulation with increasing concentrations of BTC or EPR in a BaF3 cell line that expresses both EGFR and ErbB4 (Fig. 6*A*).

We first compared the magnitude of receptor phosphorylation stimulated by EPR and BTC by plotting receptor phosphorylation relative to the maximal phosphorylation stimulated by BTC (Fig. 6, *B* and *C*). In agreement with results presented above (Fig. 5, *A* and *C*), EPR stimulated higher saturated levels of EGFR phosphorylation than BTC, whereas BTC activated greater ErbB4 phosphorylation than did EPR (Fig. 6, *A* and *C*). However, the magnitude of these differences was much less in the EGFR+ErbB4 cell line compared with the differences in phosphorylation that we observed between the cell lines expressing EGFR and ErbB4 individually (Fig. 5, *A* and *C*).

Next, we compared the sensitivities of EGFR and ErbB4 with BTC and EPR by identifying the growth factor concentrations required for half-maximal receptor phosphorylation. Half-maximal EGFR activation occurred at a BTC concentration of approximately 35 ng/ml, whereas half-maximal ErbB4 activation occurred at a BTC concentration of approximately 5 ng/ml (Fig. 6*B*; Table I). In contrast, half-maximal EGFR activation occurred at an EPR concentration of approximately 320 ng/ml, whereas half-maximal ErbB4 activation occurred at an EPR concentration of approximately 790 ng/ml (Fig. 6*C*; Table I).

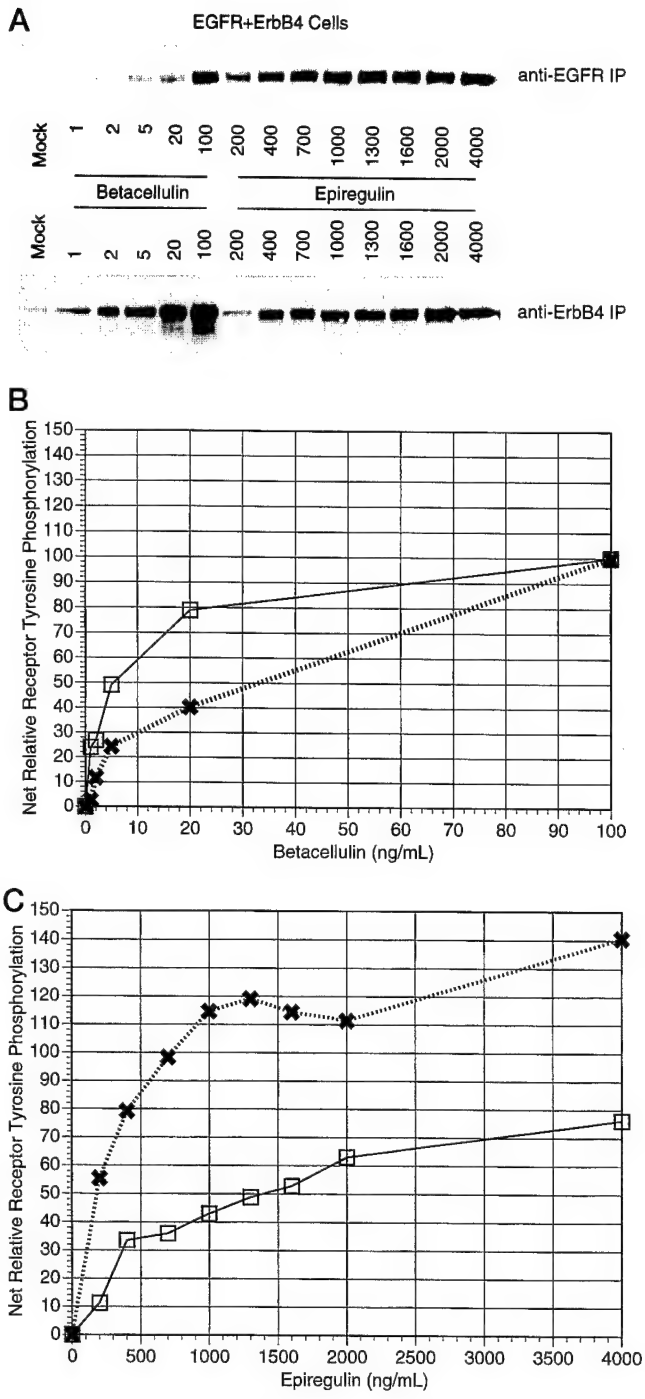

FIG. 6. **EPR and BTC dose response in BaF3 cells expressing both EGFR and ErbB4.** *A*, BaF3/EGFR+ErbB4 cells were stimulated with increasing concentrations of betacellulin or epiregulin or were mock stimulated with phosphate-buffered saline (*Mock*) as described previously (7, 14). EGFR or ErbB4 was immunoprecipitated as indicated using specific antireceptor antibodies and separated by SDS-PAGE as described previously (7, 14). Tyrosine phosphorylated ErbB family receptors were detected and visualized by immunoblotting using the 4G10 monoclonal antiphosphotyrosine antibody as described previously (7, 14). *B* and *C*, antiphosphotyrosine immunoblot images were scanned on a Hewlett-Packard ScanJet 3p flatbed scanner set for 600 dpi optical resolution. Images were cropped using Adobe Photoshop and receptor tyrosine phosphorylation was quantified using NIH Image. Net receptor tyrosine phosphorylation was calculated by subtracting the receptor tyrosine phosphorylation exhibited by mock stimulated cells. Tyrosine phosphorylation stimulated by BTC (*B*) or EPR (*C*) was expressed relative to the maximal tyrosine phosphorylation stimulated by BTC. ✖, EGFR; □, ErbB4.

This suggests that ErbB4 is 7-fold more sensitive to BTC than is EGFR, whereas EGFR is more than 2-fold more sensitive to EPR than is ErbB4.

Finally, these results illustrate that EGFR expression, like ErbB2 expression, shifts the EPR dose-response curve in cells expressing ErbB4. Half-maximal ErbB4 phosphorylation in a CEM cell line expressing ErbB4 alone occurs at an EPR concentration of 1790 ng/ml (Fig. 5*D*; Table I). In contrast, half-maximal ErbB4 phosphorylation in BaF3 cells expressing both EGFR and ErbB4 occurs at 790 ng/ml (Fig. 6*C*; Table I).

EPR Activates ErbB Family Receptor Coupling to IL3 Independence—Although EPR and BTC stimulate qualitatively identical patterns of receptor phosphorylation, these hormones are quantitatively distinct. One possible mechanism is that EPR and BTC stimulate EGFR and ErbB4 tyrosine phosphorylation at different sites. This would account for the higher levels of EGFR activation by EPR compared with BTC and the higher levels of ErbB4 activation by BTC compared with EPR. Moreover, this would also enable these hormones to couple to distinct receptor effectors and physiologic responses. Therefore, we compared EPR and BTC induction of receptor coupling with physiologic responses. BaF3 cells require interleukin-3 (IL3) for survival and for proliferation. Activation of either EGFR or ErbB2 permits survival of BaF3 cells in the absence of IL3 (7, 14). However, ErbB4 activation by either NRG or BTC is not coupled to IL3-independent survival (7,14). In BaF3 cells expressing both ErbB2 and ErbB4 together, activation by either BTC or NRG induces IL3-independent survival, presumably through ErbB2 transmodulation by ErbB4 (7, 14).

EPR, like BTC (7), induces IL3-independent survival in BaF3 cells expressing EGFR, but not in vector control BaF3 cells or cells expressing ErbB2 (Fig. 7). (The IL3-independent response of BaF3 cells expressing ErbB2 to NRG is the result of ErbB2 transmodulation by endogenous ErbB3 in these cells (14).) EPR, BTC, and NRG all induced IL3 independence in cells co-expressing ErbB2 and ErbB4 (Fig. 7). This implies that BTC and NRG are functionally equivalent. However, the response to BTC and NRG is greater than the response to EPR, which may reflect subtle functional differences between BTC and EPR.

DISCUSSION

We previously demonstrated that the EGF family of peptide growth factors can be divided into three distinct functional groups (8) (Fig. 8). The first group consists of EGF, TGF-α, and amphiregulin. These hormones bind and activate only the EGFR, but they can activate the other three ErbB family receptors *in trans* via heterodimerization with the EGFR. The second group consists of NRG and NRG2, which bind ErbB3 and ErbB4 and transmodulate EGFR and Neu via the binding receptors. The third group consists of BTC, which binds and activates both EGFR and ErbB4. Recent data suggests that heparin-binding EGF-like growth factor may also bind and activate EGFR and ErbB4, which would make heparin-binding EGF-like growth factor a member of this group as well (22).

Although EPR activates both EGFR and ErbB4, the interactions of EPR with these two receptors appear to be quite different. Compared with BTC, EPR stimulates higher levels of EGFR phosphorylation and lower levels of ErbB4 phosphorylation. Whereas both EPR and BTC stimulate EGFR and ErbB4 homodimerization and signaling, the geometry of the receptor dimers induced by EPR and BTC may be subtly different. The alignment of the kinase domain of one receptor molecule of a receptor homodimer with the autophosphorylation site of the other receptor molecule following EPR stimulation could be different from this alignment following BTC stimulation, affecting the cross-phosphorylation within receptor dimers. Al-

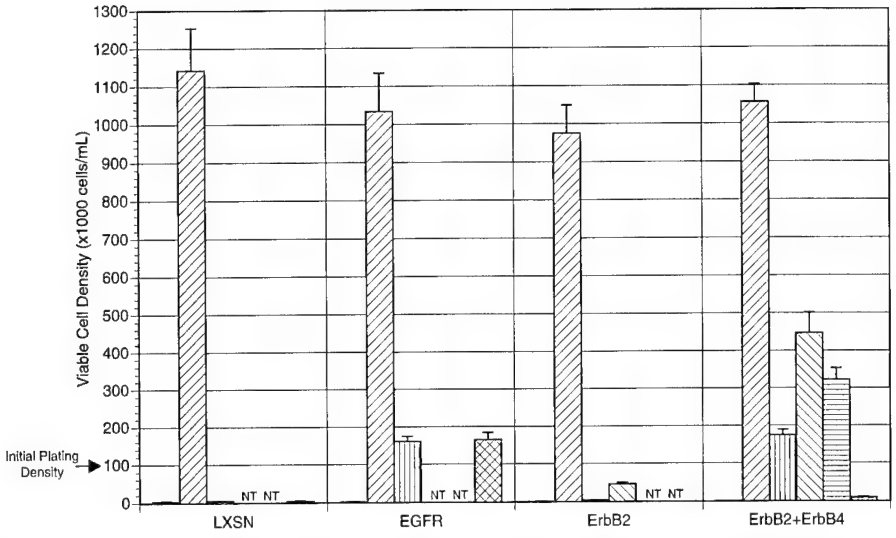

FIG. 7. **EPR stimulation of IL3-independent responses in BaF3 cells expressing various ErbB family receptors.** The IL3-independent responses of BaF3/LXSN (vector control), BaF3/EGFR, BaF3/ErbB2, and BaF3/ErbB2+ErbB4 cells to EPR stimulation were assayed as described earlier (7, 14). Briefly, cells were seeded in duplicate or triplicate at an initial density of 100×10^3 cells/ml in medium lacking IL3 (□), containing IL3 (▨), or lacking IL3 but supplemented with 10 ng/ml EPR (▥), 10 ng/ml BTC (▤), 10 ng/ml synthetic NRGβ (▧) or 10 ng/ml TGF-α (▩). After seeding, samples were taken every 24 h, and the viable cell density was calculated by staining cells with trypan blue and counting them in a hemocytometer. Samples were taken until the viable cells reached a saturation density. The mean and standard error densities for three to seven trials are shown. *NT*, not tested. Unpublished work has demonstrated that 10 ng/ml BTC induces saturated amounts of IL3 independence in a variety of BaF3 cell lines, whereas 10 ng/ml EPR induces saturated amounts of IL3 independence in the BaF3/EGFR cell line.

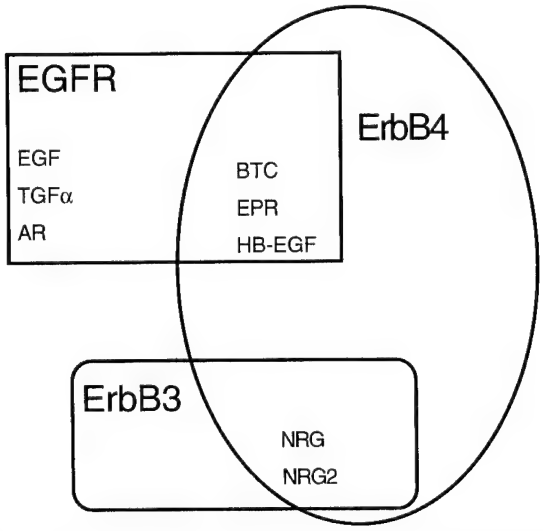

FIG. 8. **Venn diagram illustrating the activities of EGF family hormones.** The three functional groups of EGF family hormones are illustrated using a Venn diagram. The diagram is based on data presented in this work and data from Refs. 7, 8, 14, and 22.

ternatively, ligand-induced changes in the conformation of the receptor kinase domains might be different when the receptors are activated by BTC and EPR. Therefore, BTC and EPR may differentially stimulate receptor kinase activity. In either scenario, BTC and EPR could stimulate receptor autophosphorylation on different tyrosine residues, which could also be reflected in differences in gross levels of receptor phosphorylation. In this manner BTC and EPR could differentially modulate receptor coupling to signaling effectors and physiologic responses.

Another difference between EPR and BTC is that while EGFR is much more sensitive than ErbB4 to EPR, EGFR is less sensitive than ErbB4 to BTC. This suggests that although the affinity of EPR for EGFR is higher than the affinity for ErbB4, the affinity of BTC for EGFR is lower than the affinity for ErbB4. This too suggests that BTC and EPR have distinct biological functions, even in cells with identical patterns of ErbB family receptor expression.

Another important aspect of EPR function is the observation that the sensitivity of ErbB4 for EPR and the magnitude of ErbB4 activation by EPR can be modulated by the expression of other ErbB family receptors. EGFR expression increases the sensitivity of ErbB4 for EPR, suggesting that EGFR-ErbB4 heterodimers have a higher affinity for EPR than ErbB4-ErbB4 homodimers (Fig. 5, *A* and *D*; Fig. 6, *A* and *C*; Table I). Of course an alternative explanation is that the increased ErbB4 sensitivity in the presence of EGFR is due solely to EPR-induced transphosphorylation of ErbB4 by EGFR.

ErbB2 also increases the sensitivity of ErbB4 for EPR (Fig. 5, *A-D*; Table I). Because EPR does not activate ErbB2 in cells devoid of EGFR or ErbB4 (Figs. 1 and 7), the mechanism for the increased sensitivity of ErbB4 for EPR may be that ErbB2-ErbB4 heterodimers have a higher affinity for EPR than do ErbB4-ErbB4 homodimers. These results resemble observations made with NRG, which does not bind to ErbB2, binds with low affinity to cells expressing ErbB3, and binds with higher affinity to cells that express both ErbB2 and ErbB3 (12). ErbB2 expression also increases the magnitude of ErbB4 activation by EPR.

These observations that ErbB4 activation by EPR can be influenced by EGFR or ErbB2 is consistent with existing models for receptor heterodimerization and transmodulation. It has been proposed that receptor heterodimerization is mediated through low affinity hormone-receptor interactions and heterotypic receptor-receptor contacts, after which there is cross-phosphorylation by the receptor kinase domains (23). It is possible that EGFR and ErbB2 are favored over ErbB4 for dimerization with ErbB4 in the presence of EPR. Therefore, there would be greater ErbB4 dimerization in cells expressing EGFR and ErbB4 or Neu and ErbB4 than in cells expressing ErbB4 alone. This may account for the increased sensitivity of ErbB4 for EPR in the presence of EGFR or ErbB2. It is also possible that ErbB2 is a better kinase for ErbB4 than ErbB4 itself. Consequently, ErbB2 may cross-phosphorylate more ErbB4 tyrosine residues in receptor heterodimers than ErbB4 would in receptor homodimers. Similarly, ErbB2 may phospho-

rylate the same tyrosine residues as ErbB4 to a greater extent than does ErbB4. Either of these last two possibilities would account for the increased tyrosine phosphorylation of ErbB4 by EPR in the presence of ErbB2.

As this manuscript was being prepared for submission, it was reported that radiolabeled EPR can be cross-linked to EGFR and ErbB4 in human breast tumor cell lines but not to ErbB2 or ErbB3. Furthermore, EPR stimulated high levels of EGFR and ErbB4 tyrosine phosphorylation and more modest levels of ErbB2 and ErbB3 tyrosine phosphorylation (24). Because the cell lines used in these studies express at least two and in some cases all four ErbB family receptors, some caution must be used in interpreting these results. Nonetheless, these data are entirely consistent with our findings that EGFR and ErbB4 are the receptors for EPR.

To date there have been only a few clues to EPR function. EPR transcripts are not detected in normal adult mouse liver, kidney, brain, spleen, testis, or skeletal muscles. However, low levels of EPR transcripts are detectable in adult mouse lung, smooth muscle, and heart, whereas more robust EPR transcription is observed in whole embryo RNA samples from 7-day-old mouse embryos (25).[3] This implies that EPR plays a significant role in early mammalian development but only a limited role in adult tissues.

Additional hints to EPR function arise from our data suggesting that EPR is a ligand for both EGFR and ErbB4. In most contexts EGFR activation is coupled to cellular DNA synthesis and proliferation. In contrast, there is mounting evidence that activated ErbB4 is coupled to growth inhibition, differentiation, and possibly tumor suppression. NRG, a ligand for ErbB3 and ErbB4, inhibits the proliferation and stimulates the differentiation of a number of human breast tumor cell lines (26), whereas NRG implants stimulate the differentiation of the mouse mammary epithelium *in vivo* (27). BTC stimulates the differentiation of pancreatic AR42J cells into insulin-secreting cells, but EGF and TGF-α do not (28). Finally, agonistic anti-ErbB4 antibodies stimulate the differentiation of human breast tumor cell lines (29), and ErbB4 overexpression in breast cancer patients correlates with progesterone receptor expression, which is a marker for longer disease-free survival and better overall prognosis (30). Because EPR is a ligand for both EGFR and ErbB4, EPR may act as a proliferative agent in cells expressing EGFR and may act as a differentiation agent in cells that express ErbB4.

Furthermore, because EPR activation of ErbB4 is regulated by ErbB2 and activated ErbB2 appears to couple to mitogenesis and cell proliferation, the effects of EPR on cells expressing ErbB4 may be tightly linked to a balance of ErbB4 and ErbB2 expression. In cells having relatively low levels of ErbB2, EPR may have little effect because it fails to bind to ErbB4, and in cells having moderate levels of ErbB2 and high levels of ErbB4, EPR may act as a differentiation agent and inhibit cell proliferation, because the relatively high levels of ErbB4 signaling may overcome the effects of ErbB2 signaling. Finally, in cells having relatively high levels of ErbB2 relative to ErbB4, EPR may stimulate such high levels of ErbB2 signaling that the effects of ErbB4 signaling are overcome, and cell proliferation is stimulated. In sum, our data suggests that the physiologic response to EPR will be dictated by relative levels of EGFR, ErbB2, and ErbB4 expression and not just the absolute level of expression of any single ErbB family receptor.

[3] T. Komurasaki, unpublished data.

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Specificity within the EGF family/ErbB receptor family signaling network

David J. Riese II and David F. Stern*

Summary

Recent years have witnessed tremendous growth in the epidermal growth factor (EGF) family of peptide growth factors and the ErbB family of tyrosine kinases, the receptors for these factors. Accompanying this growth has been an increased appreciation for the roles these molecules play in tumorigenesis and in regulating cell proliferation and differentiation during development. Consequently, a significant question has been how diverse biological responses are specified by these hormones and receptors. Here we discuss several characteristics of hormone-receptor interactions and receptor coupling that contribute to specificity: 1) a single EGF family hormone can bind multiple receptors; 2) a single ErbB family receptor can bind multiple hormones; 3) there are three distinct functional groups of EGF family hormones; 4) EGF family hormones can activate receptors in *trans*, and this heterodimerization diversifies biological responses; 5) ErbB3 requires a receptor partner for signaling; and 6) ErbB family receptors differentially couple to signaling pathways and biological responses. BioEssays 20:41–48, 1998.

INTRODUCTION

The signaling network composed of the epidermal growth factor (EGF) family of hormones and their receptors regulates the proliferation and differentiation of many tissue types. Deregulation of this network is a significant factor in the genesis or progression of several human cancers, including neoplasms of the brain, lung, breast, ovary, pancreas, and prostate.[1,2] These observations have spurred efforts to elucidate how this signaling network is regulated and coupled to physiological responses and how regulation and coupling are disrupted in malignancies.

Efforts to characterize this signaling network also have been triggered by the observations that the EGF family peptides, called neuregulins (NRGs), play a significant role in neural development and function. Neurons produce NRGs, whereas postsynaptic cells or cells associated with neurons (glia or Schwann cell precursors) express ErbB family receptors. NRG activates these receptors through a paracrine or juxtacrine mechanism. For example, NRGs produced by motor neurons induce acetylcholine receptor subunit transcription and protein synthesis in postsynaptic muscle cells, which express ErbB2 and ErbB3 and possibly EGFR and ErbB4.[3] Furthermore, NRGs expressed from sensory neuron axons stimulate Schwann cell proliferation and may promote the differentiation of neural crest cells into Schwann cell precursors.[4] In general, patterns of ErbB3 and ErbB4 expres-

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sion differ during neural development, suggesting that these NRG receptors couple to distinct signaling pathways and cellular responses.[3] Additional functions for NRGs have been identified in mutants in which NRG signaling is disrupted.[5-8] Such mutants display defects in the peripheral nervous system, most notably a loss of cells in the cranial sensory ganglia or a misinnervation of rhombomeres by cranial sensory and motor neurons. Again, because multiple ErbB family receptors appear to be NRG effectors, there is significant interest in how these receptors couple to distinct signaling effectors and biological function.

EGF FAMILY HORMONES

There are at least eight different hormones in the EGF family: EGF itself, transforming growth factor alpha (TGF-α); heparin-binding epidermal growth factor-like factor (HB-EGF); amphiregulin (AR), also known as keratinocyte autocrine factor or colorectum-cell derived growth factor; epiregulin (EPR); betacellulin (BTC); the neuregulins (NRGs), also known as heregulins, neu differentiation factors, glial growth factors, acetylcholine receptor inducing activity, or sensory and motor neuron-derived factor; and the neuregulin-2s (NRG-2s), also known as the cerebellum-derived growth factors. Moreover, multiple NRG and NRG-2 isoforms arise from alternative transcriptional splicing.[1-4,9-11]

Most EGF family peptides are synthesized as transmembrane precursors that can be proteolytically cleaved to release the soluble form of the hormone or can function as membrane-anchored hormones in juxtacrine signaling. The soluble hormones can be as small as 50 amino acids, sharing a domain of homology that encompasses approximately 50 amino acids. The salient feature of this domain is six characteristically spaced cysteine residues that form three intramolecular disulfide linkages and define a three-loop secondary structure.[2] This domain is both required and sufficient for ErbB family receptor binding and activation; little is known about the physiological functions of the non-EGF homologous domains, which can be extensive.

Two additional proteins share limited homology with EGF family hormones. Cripto-1[12,13] and Cryptic[14] contain the six cysteine residues characteristic of EGF family hormones. However, the spacing of these residues is altered such that Cripto-1 and Cryptic completely lack the "A-loop" formed by the residues between the first and second cysteine residues, and the "B-loop" formed by the residues between the third and fourth cysteine residues is considerably smaller than this domain in other EGF family hormones. Indeed, the synthetic EGF homology domain of Cripto-1 does not activate ErbB family receptors.[15]

ERBB FAMILY RECEPTORS

There are four ErbB family receptors: epidermal growth factor receptor (EGFR, also called HER; ErbB);[16] ErbB2 (also Neu, HER-2);[17,18] ErbB3 (HER3);[19,20] and ErbB4 (HER4).[21] The human forms of these receptors range in size from 1,210 to 1,343 amino acids. They each consist of a cysteine-rich extracellular domain, a single membrane-spanning domain, and a large cytoplasmic domain composed of a tyrosine kinase domain and several tyrosine residues that are phosphorylated upon receptor activation. Ligand binding stimulates receptor dimerization and tyrosine phosphorylation at several sites that then serve to dock effector proteins and couple to physiological responses.

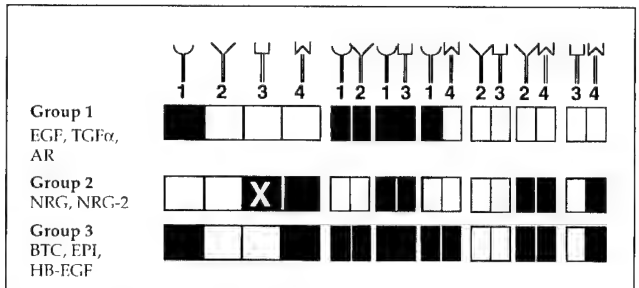

Figure 1. Three distinct functional groups of EGF family hormones. The patterns of ErbB family receptor activation stimulated by various EGF family hormones are shown for cell lines expressing ErbB family receptors singly or in pairwise combinations. Where there are quantitative variations within groups, the pattern shown applies to the prototype member listed first. A filled box indicates receptor activation, with the fill darkness indicating the intensity of receptor activation. A white "x" on a filled background indicates that the hormone binds the receptor but does not activate it. Adapted from references 10, 29, 31, 35, 36, 42, 43, and 74–83.

DIFFERENTIAL ACTIVATION AND COUPLING OF ERBB FAMILY RECEPTORS

Differential Activation of ErbB Family Receptors

A number of mechanisms contribute to the complexity and interconnectedness of the EGF family/ErbB family signaling network. These include the large number of ligands and extensive cross-interactions among the receptors.

(1) A Single EGF Family Hormone Can Bind Multiple Receptors

For example, BTC, HB-EGF, and EPR activate both the EGF receptor and ErbB4, whereas NRG and NRG-2 both bind ErbB3 and ErbB4 (Fig. 1).

(2) A single ErbB Family Receptor Can Bind Multiple Hormones

With the exception of ErbB2, which is an orphan receptor, each ErbB family receptor binds multiple hormones. EGF, TGF-α, HB-EGF, AR, BTC, and EPR bind to the EGFR. BTC,

NRG, NRG-2, HB-EGF, and EPR bind ErbB4, but only NRG and NRG-2 bind ErbB3 (Fig. 1).

(3) There Are Three Distinct Functional Groups of EGF Family Hormones

These groups are distinguished by their abilities to bind to and activate distinct sets of individual receptors (Fig. 1). The first group consists of EGF and its functional analogues TGF-α, and AR, which all bind and activate EGFR but not the other receptors. The second group consists of NRG and NRG-2, which bind ErbB3 and ErbB4. The third group consists of BTC, EPR, and HB-EGF. These hormones bind and activate both EGFR and ErbB4.

(4) EGF Family Hormones Can Activate Receptors in trans

Receptors that do not bind a particular hormone when expressed alone can be cross-activated ("transmodulated") if a binding competent receptor is also present. For example, although EGF does not bind to or activate ErbB2 expressed by itself, EGF induces the tyrosine phosphorylation of both EGFR and ErbB2 in cells expressing both receptors[22–24] (Fig. 1). Furthermore, even a kinase-inactive ErbB2 mutant can be cross-activated by EGF and EGFR.[25] Because the transmodulation of ErbB2 by the EGFR is accompanied by the formation of EGF-stimulated EGFR-ErbB2 heterodimers,[26,27] it is likely that the kinase responsible for ErbB2 transmodulation is the EGFR itself. Nonetheless, a plausible alternative is that an *src*-family kinase activated by the EGFR is involved.[28]

Analogous heterotypic interactions are now known to occur extensively among other combinations of ErbB family receptors (Fig. 1). The presence of a single hormone-binding receptor is generally sufficient for EGF family hormones to activate all other ErbB family members present. However, RTKs outside the ErbB receptor family do not cross-activate these receptors, nor can they themselves be activated in trans by EGF family hormones and ErbB family receptors. There are two notable exceptions. Although BTC activates ErbB4, BTC does not activate ErbB3 in cells expressing both ErbB3 and ErbB4 (Fig. 1).[29] Similarly, although NRG-α binds ErbB3, it does not activate either EGFR or ErbB3 in cells expressing these receptors.[30]

At a more quantitative level, there is a graded hierarchy of heteromeric interactions that may reflect differences in affinities of the various hormone-receptor-receptor complexes. For example, EGF, which binds only the EGFR, transmodulates ErbB2 more strongly than ErbB3 or ErbB4. Similarly, NRG-β, which binds ErbB3 and ErbB4, transmodulates ErbB2 more strongly than EGFR. This suggests that ErbB2 is the preferred target for transmodulation by a ligand-activated ErbB family receptor.[31–33]

Because many cell types express at least three of the four receptors, this implies that there is competition among receptors for dimerization partners. This may explain the finding that pretreatment with NRG inhibits subsequent EGF binding, suggesting that recruitment of the EGFR into complexes with ErbB3 or ErbB4 reduces the availability of free EGFR.[34] Moreover, transmodulation of the EGFR in T47D cells by NRG (via ErbB3 and ErbB4) is enhanced when ErbB2 is selectively removed.[33] However, a simple competition model does not account for the observation that down-regulation of ErbB2 *reduces* ErbB4 transmodulation by EGF (and the EGFR).[33]

The mechanism and stoichiometry of heteromeric receptor interactions have not been determined; this reflects the absence of a basic understanding about receptor oligomerization. For example, it is possible that higher order oligomers rather than dimers are the active signaling species. Furthermore, it is not known if a ligand binds to one or both receptors in a heterotypic receptor complex. Coexpression of binding and nonbinding ErbB family members in some cases enhances hormone-binding affinity,[35] whereas intracellular retention of ErbB2 reduces EGF and NRG binding by accelerating their dissociation.[36] (We use "binding" and "nonbinding" here to denote the behavior of the receptors when expressed individually.) The means by which a nonbinding receptor is recruited into a receptor complex and can modulate ligand-binding affinity are uncertain. Hypothetically, in EGFR/ErbB2 transmodulation, EGF binding may unveil a cryptic EGFR/ErbB2 interreceptor binding site and/or may stabilize EGF binding to ErbB2. Similarly, formation of EGFR-ErbB2 dimers may alter the conformation of the ErbB2 hormone-binding domain or create a composite-binding site encompassing elements from both receptors. EGF appears to bind the EGFR with a 1:1 stoichiometry,[37,38] and it has been proposed that EGF binds bivalently, with each EGF molecule binding to a high-affinity site on one EGFR molecule and a low-affinity site on another EGFR molecule.[38] Bivalent hormone binding is consistent with the observation that BTC and EGF-NRG chimeras bind both EGFR and ErbB4.[29,39] It has been proposed that hormone binding to the high-affinity binding site is required to stabilize receptor dimers and for receptor activation. Therefore, bivalent binding of a hormone molecule to a high-affinity site on one receptor ("binding" receptor) and a lower affinity site on a heterotypic ErbB family receptor molecule ("nonbinding" receptor) may be the mechanism underlying receptor heterodimerization and transmodulation. Differences in the affinity of this "lower affinity" binding site on the "nonbinding" receptor may explain the variable heterodimerization and transmodulation potential for the four ErbB family receptors. And finally, these possibilities mean that coexpression of ErbB family receptors may create novel "emergent" binding specificities.

Intriguing observations that have led to a greater understanding of ErbB family receptor heterodimerization are that

NRG induces the formation of EGFR-Neu heterodimers and that this heterodimerization is blocked by the Neu tyrosine kinase inhibitor tyrphostin AG879. Ligand-induced ErbB family receptor dimers apparently dissociate and can nucleate "secondary" (hetero)dimerization with additional receptor molecules. Moreover, receptor phosphorylation is apparently required for dissociation of the "primary" receptor dimers and for formation of "secondary" heterodimers.[40] Because receptor phosphorylation also triggers receptor internalization and degradation, the amount of receptor heterodimerization must be regulated by a number of factors, including hormone and receptor concentration, the affinity for receptor dimerization, receptor kinase activity, and rate of receptor internalization.

(5) ErbB3 Requires a Partner

A special case is the reliance of ErbB3 signaling on heteromeric interactions. Four residues in the ErbB3 tyrosine kinase homology domain diverge from the consensus tyrosine kinase sequence,[20,41] and ErbB3 has little or no associated kinase activity.[41] Although ErbB3 expressed alone binds NRGs, tyrosine phosphorylation of this receptor only occurs in the presence of additional ErbB family members. Presumably, they are required for cross-phosphorylation of ErbB3.[29,35,36,42,43]

COUPLING OF ERBB FAMILY RECEPTORS TO BIOLOGICAL RESPONSES AND SIGNALING EFFECTORS

The complex regulation of ErbB family receptor activation is significant because the four receptors couple to distinct sets of signaling effectors and biological responses.

(1) ErbB Family Receptors Have Different Signaling Specificities

Each receptor has unique signaling specificities, as first suggested by the unique hormone-independent transforming activity of overexpressed ErbB2 relative to the other receptors.[44–51] These differences reflect differential activation of signaling pathways, as was first shown to occur for EGFR and ErbB2.[46,52]

(2) ErbB Family Receptor Heterodimerization Diversifies Biological Responses

An early indication that activation of multiple ErbB family receptors increases response diversity was the finding that EGFR and ErbB2 synergize in transformation of NR6 fibroblasts.[53] Similarly, a subset of receptor combinations promotes hormone-independent or NRG-dependent transformation of NIH3T3 cells.[48,49,51] Interleukin 3 (IL-3)-independent survival or proliferation of a panel of Ba/F3 cells with defined receptor content depends on the specific combinations of

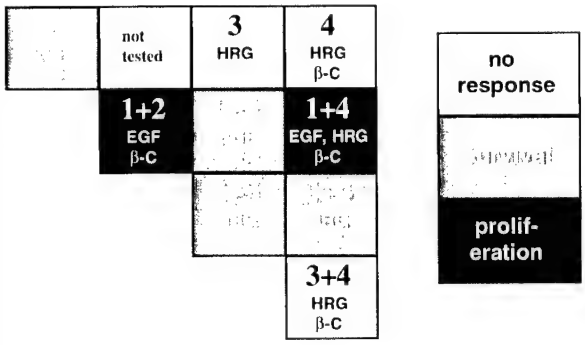

Figure 2. Responses of recombinant Ba/F3 cell lines to ErbB family receptor activation. The patterns of interleukin-3 (IL-3)-independent responses stimulated by various EGF family hormones are shown for Ba/F3 cell lines expressing ErbB family receptors singly or in pairwise combinations. A filled box indicates an IL-3-independent response, with a gray fill indicating IL-3-independent survival and a black fill indicating IL-3-independent proliferation. The hormones used to elicit these responses are indicated by name. Adapted from references 29, 43, and 80.

receptors activated (Fig. 2). In this cell background, activation of ErbB4 alone yields no measurable response, whereas activated EGFR couples to IL-3-independent survival. However, activation of EGFR and ErbB4 together leads to IL-3-independent proliferation.[29,43] Conversely, selective intracellular retention (and inactivation) of ErbB2 in T47D cells radically diminishes the extent and duration of MAP kinase activation by EGF and NRG, suggesting that MAP kinase is predominantly coupled to ErbB2 activated by transmodulation.[36]

The simplest interpretation for receptor cooperativity in induction of biological responses is that different receptors activate complementary signaling pathways. However, an alternative explanation is that receptor heteromers have unique signaling specificities. This is plausible, because in a cross-phosphorylation reaction within a heterodimer, the identity and geometry of kinase/substrate pairs differ from that for a homodimer. In NIH3T3 cells ectopically expressing EGFR and ErbB3, NRG stimulates anchorage-independent growth, whereas EGF does not, despite the fact that EGF stimulates higher levels of receptor phosphorylation than NRG. Furthermore, EGF treatment mobilizes intracellular Ca^{2+} stores, whereas NRG treatment does not.[51] Similarly, when the EGFR is activated by HB-EGF in a breast tumor cell line, the downstream signaling effector Cbl is tyrosine phosphorylated and complexes with the EGFR. However, when EGFR is activated by NRG through transmodulation by ErbB3 or ErbB4, Cbl is not tyrosine phosphorylated, nor does it complex with the EGFR, despite the fact that HB-EGF and NRG stimulate similar levels of EGFR phosphorylation.[33]

These data suggest that distinct sets of receptor tyrosine residues become phosphorylated in response to different stimuli, resulting in differential coupling to signaling effectors and biological responses.

(3) ErbB Family Receptors Couple to Distinct Cellular Signaling Effectors

Although it is clear that ErbB family receptors differentially couple to biological responses, the specific substrates and pathways associated with these different responses have not been elucidated. A number of receptor effectors have been identified, a few of which may be receptor-specific.[1,31,36,46,52,54–59] For example, Cbl is activated by and complexes with the EGFR but not the other ErbB family receptors.[60] Similarly, the CHK Csk-homologous kinase binds to ErbB2 but does not bind EGFR, ErbB3, or ErbB4.[61]

CONCLUSIONS

Emerging evidence shows that the extensive cross-interactions revealed by tissue culture analysis of the EGF system has profound importance in vivo. This is manifested by the striking phenotypic similarities of mice with homozygous disruptions of the ErbB2, ErbB4, and NRG genes.[5–8] These three gene disruptions are all embryonically lethal at day 10.5 postcoitum and induce overlapping but not identical defects in the nervous system. Significantly, these animals all lack the trabecular extensions of the ventricular myocardium, which results in lethality due to cardiac malfunction. ErbB2 and ErbB4 are expressed in the myocardium, whereas NRG is expressed in the endocardium. The disruption phenotypes show that paracrine activation of both ErbB2 and ErbB4 by NRG is required for proper myocardial development and verifies the importance of transmodulation of ErbB2 in a physiological response to NRG.[4,62]

A number of open questions regarding the regulation and coupling of this signaling network remain. For the hormones, there is little understanding of the role of the non-EGF homologous domains, which can be quite large and contain a number of recognizable motifs. For example, some of the differentially spliced NRG isoforms include an immunoglobulin (Ig)-like domain, a glycosylation-rich spacer domain, a cysteine-rich "sensory and motor neuron-derived factor" domain, and a variant kringle domain.[9,63] Although these domains are not required for activation of ErbB3 or ErbB4, they may regulate hormone-receptor interactions in a quantitative manner. In fact, mutant mice homozygous for a mutant NRG gene containing a disruption in the Ig-like domain die during embryogenesis and exhibit neurologic and cardiac defects similar to those observed in mice homozygous for complete disruption of the NRG gene.[5,8] Although this implies that the Ig-like domain is required for ErbB family receptor activation, another possibility is that this domain is required for NRG stabilization or presentation by extracellular matrix in vivo. In a similar vein, only a little more is known about regulation of hormone-receptor interactions by heparin-sulfate proteoglycans (HSPGs). NRG, HB-EGF, and AR bind HSPGs, and HSPGs regulate the interactions of HB-EGF and AR with the EGFR.[64] HSPGs may regulate ligand binding by acting as low-affinity receptors for AR and HB-EGF, increasing the local concentration of these hormones.

EGF family hormones are initially synthesized as membrane-anchored precursors that are subsequently cleaved to release soluble hormone. Experiments using mice homozygous for disruptions in the ErbB4 and NRG genes suggest that activation of ErbB family receptors by membrane-anchored hormones on an adjacent cell or tissue (juxtacrine regulation) is required for some developmental processes. It is unclear, however, whether the membrane-anchored and soluble forms of EGF family hormones stimulate identical patterns of receptor tyrosine phosphorylation. Steric and conformational constraints on the membrane-anchored hormones might restrict their activities. Several of the membrane-anchored hormone precursors possess significant cytoplasmic tails. During juxtacrine receptor activation the membrane-anchored hormones also may act as receptors, coupling to signaling effectors and physiological changes in response to ErbB family receptor binding. This possibility has stimulated efforts to identify proteins that interact with the cytoplasmic domains of EGF family prohormones. The cytoplasmic domain of TGF-α has been shown to associate with an uncharacterized protein kinase activity as well as a p86 cytoplasmic protein. However, the identities of the protein kinase and the p86 cytoplasmic protein have yet to be determined, and the regulation of these interactions has yet to be defined.[65,66]

Another emerging field of study is the role of ErbB family receptors as effectors for stimuli independent of EGF family hormones,[67] much as nonreceptor protein tyrosine kinases serve as signaling effectors for receptors lacking kinase activity. Treatment of HeLa cells with short-wavelength ultraviolet light (UVC) stimulates EGFR and p42 MAP kinase phosphorylation and induces c-*fos* and c-*jun* transcription. These responses are not seen in HeLa cells ectopically expressing a dominant-negative EGFR mutant.[68] It has been proposed that one mechanism by which UV induces cellular responses is through the induction of reactive oxygen species (ROS), such as hydrogen peroxide. Therefore, it is not surprising that in vascular smooth muscle cells hydrogen peroxide also stimulates the phosphorylation of EGFR and MAP kinases as well as the formation of complexes containing Shc, Grb2, SOS, and EGFR.[69] Surprisingly, EGF stimulates hydrogen peroxide generation in A431 human carcinoma cells, and eliminating hydrogen peroxide with catalase reduces EGFR tyrosine phosphorylation in response to EGF. It has been proposed that hydrogen peroxide inhibits a protein phosphatase specific for the EGFR and that this

inhibition is required for the maintenance of EGFR in its phosphorylated state.[70] Therefore, cellular stress, which in some cases is accompanied by ROS release, may be coupled to the MAP kinase signaling pathway through EGFR and perhaps other ErbB family receptors as well.

There is also increasing evidence that G protein-coupled serpentine receptors (GPCR) also regulate ErbB family receptor signaling. The GPCR ligands thrombin, endothelin-1, and lysophosphatidic acid all activate Neu, EGFR, Shc, Grb2, and the ERK1 and ERK2 MAP kinases in Rat-1 fibroblasts. Furthermore, activation of these signaling pathways by GPCR ligands is disrupted in Rat-1 cells expressing a dominant negative EGFR mutant or in Rat-1 cells preincubated with the EGFR antagonist tyrphostin AG1478.[71] The mechanism for ErbB family receptor phosphorylation in response to GPCR ligands is still unclear, although it has been proposed that src family kinases may be involved. An interesting twist is that the purified EGFR phosphorylates the $G_{s\alpha}$ subunit of the heterotrimeric G protein complex in vitro and that phosphorylated $G_{s\alpha}$ displays increased GTPase activity and greater GTPγS binding capacity and that phospho-$G_{s\alpha}$ augmented adenyl cyclase activity in S49 cyc⁻ cell membranes.[72,73] Because EGF stimulates cAMP accumulation in the heart via $G_{s\alpha}$,[72] cross-talk between ErbB family receptors and the GPCR signal transduction pathway may be bidirectional.

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